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Authors: HOWARTH, J. A., HOKAMA, Y., and AMERAULT, T. E.

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# THE MODIFIED CARD AGGLUTINATION TEST: AN ACCURATE TOOL FOR DETECTING ANAPLASMOSIS IN COLUMBIAN BLACK-TAILED DEER

J. A. HOWARTH and Y. HOKAMA, Department of Epidemiology and Preventive Medicine, School of Veterinary Medicine, University of California, Davis, California 95616, USA

T. E. AMERAULT, U.S. Department of Agriculture, Agricultural Research Service, Animal Parasitology Institute, Beltsville, Maryland 20705, USA

*Abstract:* Inoculation of susceptible calves confirmed that the modified card agglutination test accurately detected the anaplasmosis infection status of each of 35 Columbian black-tailed deer (*Odocoileus hemionus columbianus*). *Anaplasma marginale*, and specific antibodies, were demonstrated only in calves which received blood from deer that were positive by the card test. The modified card agglutination testing of deer serum was performed in the manner recommended for testing cattle serum with bovine-origin antigen and bovine serum factor.

## INTRODUCTION

Any program for the control or eradication of anaplasmosis in the continental United States would be influenced or even nullified by the persistent reservoir of *Anaplasma marginale* infection in nonbovine species. An accurate, simple, and inexpensive procedure is therefore required for detecting the *A. marginale* carrier state in wildlife species.

The most sensitive system for detecting clinical or latent anaplasmosis in any animal species is inoculation of splenectomized susceptible bovines with freshly drawn blood from suspect animals. This procedure is time-consuming and costly, particularly if a test bovine is required for each blood sample to be tested. It is common practice to pool the blood of several animals for inoculating a single splenectomized bovine, but the results obtained must be interpreted with caution. A positive result (production of anaplasmosis) merely indicates infection of one or possible more of the donor animals. Then the testing must be repeated on a one-to-one donor-to-recipient basis. Further, uniformly negative results with pooled blood samples[15,16] can be highly significant only if the donor blood is sufficient and is handled so as to assure survival of *A. marginale* between collection and inoculation.[15]

An interference phenomenon between anaplasmosis and eperythrozoonosis in splenectomized calves has led some investigators to use intact calves for anaplasmosis studies.[5,6] Experimental infections in intact calves have longer incubation periods, produce lower levels of parasitemia, and must be confirmed by the production of specific antibodies.

Complement fixation (CF), capillary tube agglutination (CA), modified card agglutination (MCA), and indirect fluorescent antibody (IFA) tests have been developed for the detection of anaplasmosis in cattle.[2,10,13,18] The accuracy of these test methods is widely accepted and they have been used in control programs necessitating the identification of carrier cattle. These tests have given generally unreliable and disappointing results when used for detecting *A. marginale* antibodies in the sera of animals other than the domesticated bovine.

Table 1 summarizes the available information on the accuracy of the CF and CA tests applied to sera of American wildlife species where the *A. marginale*

TABLE 1. Published data on CF and CA anaplasmosis testing of sera from American game animals.

| Test | Species | *A. marginale* infectivity | No. tested | Incorrect results | Literature reference |
|---|---|---|---|---|---|
| CF | Black-tailed deer[1] | Uninfected | 5 | 0 | 14 |
| CF | Black-tailed deer | Uninfected | 18 | 3 | 6 |
| CF | White-tailed deer[2] | Uninfected | 5 | 2 | 7 |
| CF | White-tailed deer | Uninfected | 147 | 65 | 9 |
| CF | Mule deer[3] | Uninfected | 21 | 2 | 8 |
| CF | Mule deer | Uninfected | 65 | 1 | 7 |
| CF | Mule deer | Uninfected | 10 | 2 | 19 |
| CF | Mule deer | Uninfected | 31 | 2 | 15 |
| CF | Elk[4] | Uninfected | 72 | 47 | 8 |
| CF | Elk | Uninfected | 12 | 12 | 17 |
| CF | Elk | Uninfected | 162 | 90 | 7 |
| CF | Pronghorn antelope[5] | Uninfected | 10 | 10 | 8 |
| CF | Pronghorn antelope | Uninfected | 145 | 144 | 7 |
| CF | Bighorn sheep[6] | Uninfected | 8 | 4 | 8 |
| | 54% False positive results | | Total 711 | 384 | |
| CF | Black-tailed deer | Infected | 7 | 7 | 14 |
| CF | Black-tailed deer | Infected | 12 | 12 | 3 |
| CF | Black-tailed deer | Infected | 3 | 3 | 4 |
| CF | Black-tailed deer | Infected | 23 | 18 | 6 |
| CF | White-tailed deer | Infected | 64 | 0 | 9 |
| CF | Mule deer | Infected | 28 | 9 | 8 |
| CF | Elk | Infected | 69 | 13 | 8 |
| CF | Bighorn sheep | Infected | 24 | 1 | 8 |
| CF | Bison[7] | Infected | 132 | 0 | 16 |
| | 17% False negative results | | Total 362 | 63 | |

TABLE 1 (Continued)

| Test | Species | *A. marginale* infectivity | No. tested | Incorrect results | Literature reference |
|---|---|---|---|---|---|
| CA | Black-tailed deer | Uninfected | 81 | 1 | 6 |
| CA | White-tailed deer | Uninfected | 147 | 0 | 9 |
| CA | Mule deer | Uninfected | 29 | 0 | 8 |
| CA | Elk | Uninfected | | 0 | 8 |
| CA | Pronghorn antelope | Uninfected | | 0 | 8 |
| CA | Bighorn sheep | Uninfected | | 1 | 8 |
| | <1% False positive results | | Total 257 | 2 | |
| CA | Black-tailed deer | Infected | 23 | 10 | 6 |
| CA | White-tailed deer | Infected | 64 | 5 | 9 |
| CA | Mule deer | Infected | 18 | 16 | 8 |
| CA | Elk | Infected | 10 | 1 | 8 |
| CA | Pronghorn antelope | Infected | 16 | 3 | 8 |
| CA | Bighorn sheep | Infected | 16 | 6 | 8 |
| | 28% False negative results | | Total 147 | 41 | |

1. *Odocoileus hemionus columbianus*; 2.*Odocoileus virginianus dacotensis*; 3. *Odocoileus hemionus hemionus*; 4. *Cervus canadensis canadensis*; 5. *Antilocapra americana americana*; 6. *Ovis canadensis canadensis*; 7. *Bison bison*.

infectivity of each wild animal was confirmed by animal inoculation. As shown, the CF test gave false positive results with 384 of 711 (54%) sera where known uninfected wildlife species were tested. It also gave false negative results with 63 of 362 sera of wildlife species known to be infected (17% inaccuracy).

The CA test gave false positive results for only 2 of 257 (less than 1%) sera of uninfected wildife species, but was 28% inaccurate when applied to 147 sera of wildlife species known to be infected, thus giving false negative results for 41.

The sera of 1505 African game animals comprising 19 herbivorous species were screened for antibodies to *A. marginale* by both IFA and CA tests. Positive results were more prevalent in areas where antelope grazed in the vicinity of cattle herds not dipped for ticks, but the validity of the results was not confirmed by inoculation of susceptible cattle.[12]

When the original card test[1] was applied to the sera of known uninfected wildlife species, false positive results were obtained with 5 of 18 black-tailed deer (*Odocoileus hemionus columbianus*) and 22 of 31 mule deer (*Odocoileus hemionus hemionus*) for a combined 55% inaccuracy.[6,15] The original card test gave false negative results with the sera of 12 of 23 (52%) known infected black-tailed deer.[6]

The MCA test[2] with the added reagent of bovine serum factor (BSF) gave no false positive reactions when used to test the sera of 132 uninfected American bison. Since no infected bison were available, no positive control tests were made.

Indirect fluorescent antibody titers for anaplasmosis were obtained on sera of 50 African antelope of 5 species, but the data presented[11] do not allow interpretation of the results.

This report presents results of the MCA test for *A. marginale* antibodies applied to sera of Columbian black-tailed deer. The anaplasmosis infection status of each deer was verified by calf inoculation.

## MATERIALS AND METHODS

### Study Areas

The sources of deer blood samples were two areas about 80 km apart. One area was the University of California Field Station at Hopland, in Mendocino County. This station, nearly 2,800 ha of hill and mountain grazing land used for sheep husbandry, also has a large resident population of black-tailed deer. Seventeen deer were collected at the Hopland Station during the period of 1969 to 1971, and 10 were collected in December, 1974.

The second study area, a privately owned cattle ranch in Tehama County, California, is a winter range for a migratory herd of black-tailed deer. Eight deer were collected on this ranch in February, 1975.

### Collection Procedure

Ten deer were trapped alive, and blood was obtained by jugular venipuncture. The other 25 deer were shot in the head or neck, and blood drawn immediately by cardiac puncture. A portion of the blood from each deer was placed in tubes and permitted to clot for serum separation, while the remainder was mixed with heparin to prevent clotting.

### Experimental Animals

Holstein steer calves 2 to 3 months old were obtained from a single source. The calves were not splenectomized and were negative to the CF test before inoculation with deer blood.

Each calf was inoculated subcutaneously with 15 to 70 ml of blood from a single deer. The time between blood collection and calf inoculation seldom exceeded one hr.

Inoculated calves were maintained in flyproof enclosures. Beginning 14 days after inoculation, blood was obtained from the calves 3 times per week for *A. marginale* body detection and once per week for serologic studies. All calves were maintained until transmission of anaplasmosis was demonstrated or at least 100 days post-inoculation.

### Serologic Procedure

The CF test was conducted in the standard manner at the U.S. Department of Agriculture, Animal and Plant Health Inspection Service, Veterinary Services Laboratory, Beltsville, Maryland. Serum samples from calves were frozen immediately after collection and shipped frozen to the testing laboratory. Serum samples positive at a 1/5 serum dilution to the CF test were titrated.

Clotted blood samples from deer were held for 24 hrs at room temperature (21-27 C) before serum was separated by centrifugation. Serum was then stored in individual vials at —65 C for periods of one month to 6 years. Sera were thawed and held for 24 hrs at room temperature before the MCA test was performed.

The MCA test was conducted with 0.03 ml of deer serum plus 0.03 ml of BSF and 0.014 ml of buffered antigen. The card rotator was set for 105-110 rotations per min, and a thermometer was placed on the working area to assure a temperature of 21-27 C. BSF and antigen were warmed to room temperature before the tests, and all results were read after 4 min on the card rotator.

### Hematological Methods

*A. marginale* body counts were made on blood smears stained by the Giemsa method. The percentage of red blood cells containing organisms was based on examination of several hundred in numerous microscopic fields.

## RESULTS

Blood from 35 black-tailed deer was inoculated into individual test calves. Table 2 shows that 21 of them were infected with *A. marginale*. The primary criterion for infection was demonstration of the parasite in the erythrocytes of its respective test calf. In each instance of parasitemia, specific antibodies were detected by the CF test.

One calf (donor deer 926) produced CF antibody although no *A. marginale* bodies were demonstrated in blood smears. A splenectomy was therefore performed and a parasitemia was detected 12 days later, confirming the infection.

Serum from each deer was tested for antibodies to *A. marginale* by the MCA test. The results are presented in Table 2. Calf inoculation as the standard for comparison indicated that the MCA test accurately identified each of the 21 infected and 14 uninfected deer.

Since previous studies[6] had demonstrated a high prevalence of infection in mature black-tailed deer, fawns were sampled to assure adequate numbers of negative sera. Each fawn proved negative to the MCA test, and each was uninfected as shown by calf inoculation. No antibodies were present in the serum of fawn w42 even though it was nursing doe 6805, which was infected and serologically positive.

## DISCUSSION

Since wildlife are often widely dispersed and geographically removed from conventional laboratory facilities, the collection and preservation of tissue samples poses special problems. Obtaining a reasonable number of samples from wildlife species often keeps an investigator for long periods in remote areas having minimal facilities. The samples collected must either be preserved for later testing at a conventional laboratory or be processed without sophisticated equipment.

The required reagents for the MCA test are minimal in number and available in a highly stable form. The other supplies used to perform the test are inexpensive and disposable, while the essential equipment consists of a small portable centrifuge and card rotator, both of which can be powered by an automotive battery or conventional electrical current.

Storage of deer sera at —65 C for periods of one month to 6 years did not alter the accuracy of the MCA test results.

Although the results presented pertain to only one nonbovine species, it is quite likely that the MCA test will prove valuable in investigating anaplasmosis in other

TABLE 2. Correlation of anaplasmosis MCA test results with the results of calf inoculation for blood samples collected from 35 Columbian black-tailed deer.

| Deer | | | Calves | | | Area collected in California |
|---|---|---|---|---|---|---|
| Deer # | Deer age (years) | (MCA) | Inoc.* | CF | ** | |
| 926 | 3 | + | + 110 | + | 28 | |
| 927 | 10 | + | + 22 | + | 29 | |
| 928 | 2 | + | + 22 | + | 36 | Hopland Field Station |
| 929 | 3 | + | + 32 | + | 63 | Range deer |
| 930 | 1 | — | — | — | | Mendocino County |
| 931 | 2 | — | — | — | | 1969-1971 |
| 932 | 1 | + | + 32 | + | 91 | |
| 933 | 1 | — | — | — | | |
| 953 | 2 | + | + 22 | + | 28 | |
| 954 | 2 | + | + 28 | + | 28 | |
| 955 | 1 | + | + 28 | + | 35 | |
| 956 | 3 | + | + 35 | + | 42 | |
| 957 | 2 | + | + 35 | + | 49 | |
| 958 | 7 | + | + 28 | + | 28 | |
| 1086 | 1 | + | + 33 | + | 36 | |
| 1089 | 2 | + | + 51 | + | 33 | |
| 1090 | 8 | + | + 41 | + | 67 | |
| w40 | <1 | — | — | — | | |
| w41 | <1 | — | — | — | | |
| w42 | <1 | — | — | — | | |
| y64 | <1 | — | — | — | | Hopland Field Station |
| y65 | <1 | — | — | — | | Deer in captivity |
| y66 | <1 | — | — | — | | Mendocino County |
| y67 | 2 | — | — | — | | December, 1974 |
| 350 | 3 | + | + 40 | + | 26 | |
| 498 | 4 | + | + 40 | + | 19 | |
| 6805 | 3 | + | + 40 | + | 19 | |
| 1 | 4 | + | + 28 | + | 15 | |
| 2 | 4 | + | + 28 | + | 21 | |
| 3 | <1 | — | — | — | | Dye Creek Ranch |
| 4 | <1 | — | — | — | | Migratory deer |
| 5 | 8 | + | + 28 | + | 15 | Tehama County |
| 6 | <1 | — | — | — | | February, 1975 |
| 7 | 1 | + | + 35 | + | 15 | |
| 8 | <1 | — | — | — | | |

* = Day post-inoculation when *A. marginale* bodies were demonstrated.

** = Day post-inoculation when positive test result was obtained.

wildlife species. With the addition of those factors contained in BSF, the card test as performed with bovine serum became an accurate means of detecting the *A. marginale* carrier state of Columbian black-tailed deer.

The BSF used supplied a standardized level of bovine complement and conglutinin to the test system. With these factors added to the test system, antigen-antibody complexes that were probably already formed aggregated to form visible clumps. Without these factors they might not aggregate, giving many false negative reactions as reported previously.[6] Since bovine complement and conglutinin must be present for visible aggregation to take place, the card reaction appeared to be a conglutination reaction rather than an agglutination reaction.

The false positive reactions encountered in previous tests of deer serums were not found in the present study. We believe the reason is that the samples were held at room temperature for 48 hrs before testing. During this holding period nonspecific agglutinins were apparently inactivated, eliminating false positive reactions.

Properly conducted, the MCA test is rapid, accurate and economical, though certain precautions should be recognized. A known positive and negative control sample should always be checked before unknown samples are tested. When not in use, antigen and BSF should be stored below 5 C but not frozen. BSF should not be used later than 3 hrs after it has been reconstituted. The MCA test should be performed at an ambient temperature of 21-27 C, and the results read and recorded immediately. Prolonged manipulation or allowing the card to stand before reading and recording can lead to erroneous results.

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