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Comparisons of the Complement-Fixation and Indirect Fluorescent Antibody Reactions in the Detection of Bovine Babesiasis

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SUMMARY

Complement-fixation (CF) and indirect fluorescent antibody (IFA) antigens were prepared from *Babesia bigemina* isolates obtained in Texas. These serologic procedures were evaluated on 130 serum samples sequentially collected from 5 *B bigemina*-infected mature cattle, beginning on the day of exposure and continuing for 175 days thereafter.

Both tests were effective in detecting specific antibodies for the first 84 days of infection, with 57 of 60 (95%) serums tested being positive on the CF test and 57 of 57 (100%) tests being positive to the IFA test. During the interval from 98 to 175 days, 24 of 60 (40%) of the serums tested were positive with the CF test, and 53 of 56 (95%) were positive with the IFA test.

During the first 84 days, a similar linear regression occurred in both CF and IFA serum titers, but after 98 days the IFA regression flattened out, whereas the CF titers decreased below the sensitivity threshold in 60% of the serums tested.

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Babesiasis (piroplasmosis, redwater, Texas fever) in years past was a disease of major importance in the southern states. The causative agent, originally identified as *Babesia bigemina*, was limited to those areas where ticks *(Boophilus)* occurred.[8] With the eradication of *Boophilus* in the United States in the early 1940's, babesiasis ceased to be a problem.

Probably as the result of illegal livestock movement, the incursion of *Boophilus* from Mexico has been increasingly common in recent years. On 2 occasions, babesiasis was diagnosed in association with *Boophilus* infestation of US cattle.[a] In both instances, these cases occurred near the Texas-Mexican border, in the permanent quarantine zone. In the 1st instance, the disease was identified by examining stained thin blood smears and confirmed by subinoculation into splenectomized calves. In the 2nd instance, a positive serologic response to *B bigemina* antigen was observed, and infection was confirmed by subinoculation of whole blood from the suspect cow into a splenectomized calf.

The potential for natural transmission of babesiasis is limited to the prevalence of *Boophilus*, but these ticks are known to occur sporadically along the Texas-Mexico boundary, and the need for dependable serologic diagnosis has been frequently expressed.

A wide variety of serologic tests have been described for the diagnosis of bovine babesiasis, including complement-fixation (CF), gel precipitation, fluorescent antibody tests (both direct and indirect), indirect hemagglutination, and agglutinating tests.[4,7,10-12]

[a] These isolations were made by the authors, but the findings were not published.

In 1962, Mahoney[4] prepared CF antigens from the blood of cattle with severe *B bigemina* parasitemia. This test was successfully used in epizootiologic studies, but it was discovered that CF antibody response decreased below detectable limits within 4 to 5 months after infection, even though it was highly effective in the early stages.[5] Todorovic et al[13] reported the adaptation of the CF test to a microsystem which was equally effective in the detection of specific antibodies.

The indirect fluorescent antibody (IFA) test was used in the detection of specific *B bigemina* antibodies.[2,3,7] High and persistent titers in artificially infected cattle were reported. This test was used successfully with *B bigemina, Babesia argentina, Babesia rodhaini, Babesia cervi, Babesia major,* and *Babesia divergens* by a number of workers.[2,3,7,11] Some low-level cross reactions occur between *B argentina* and *B bigemina,* but homologous titers are usually sufficiently higher to allow species differentiation.[4]

The CF and IFA tests have been adapted for use in Texas and are compared in these trials to determine the relative merits of each in detecting *B bigemina* antibodies.

Materials and Methods

Babesia bigemina infections were produced in 5 adult Hereford cattle, in excess of 5 years of age, by the subcutaneous inoculation of 5 ml *B bigemina* stabilate. The stabilate was prepared by mixing, in equal parts, packed washed erythrocytes (with a 2% *B bigemina* parasitemia) and 4 *M* dimethyl sulfoxide. The mixture was then immediately frozen in liquid nitrogen and stored until used. Following exposure, blood samples were taken twice weekly to determine packed red cell volume (PCV) and to make thin Giemsa-stained blood smears for the determination of relative parasitemia. At 4- to 7-day intervals, cell-free serum was collected and frozen at −20 C for future testing with the CF and IFA techniques.

Serums were sequentially collected from all cattle for a 175-day period after exposure, and in most instances were tested, using both the CF and IFA techniques. Serum titers of 1:5 or over were considered positive for the CF test, and titers of 1:40 and over were considered positive for the IFA technique. Twofold serial dilutions were made in each instance, beginning with a 1:5 dilution.

Test antigens were prepared from *B bigemina* isolates obtained from cattle with naturally occurring infection in Texas, maintained by passage in splenectomized calves, and stored in liquid nitrogen until used.

Complement-Fixation—The CF testing procedure followed the basic procedure as used in the anasplasmosis CF test,[1] using a *B bigemina* antigen, prepared according to techniques described by Mahoney,[4-6] with some modification. Antigen was prepared from the blood of a calf having 18% PCV and 22% *B bigemina* parasitemia. Plasma was removed and erythrocytes were washed twice in sterile saline solution (0.85% NaCl) and lysed with 0.35% NaCl as described.[6] The sediment was collected following centrifugation for 30 minutes at 8,000 × g. This sediment was washed 1 time in isotonic saline solution, suspended in saline solution, and ground in a Tenbroeck tissue grinder. The antigen was titrated against known *Babesia*-positive and -negative serum and used in 2-unit amounts in a microsystem CF test.[18] Complement and amboceptor were used in 2 unit amounts. All tests were run against known negative and positive control serum.

Indirect Fluorescent Antibody Antigen Preparation—The IFA antigen was prepared from *B bigemina* parasitized erythrocytes, using modifications of the methods described by Leeflang and Perie[8] and Ross and Lohr.[7] The *B bigemina* was passed twice in splenectomized calves. Both calves were given intramuscular injections of 10 mg of dexamethasone[b] each day for 3 days after inoculation. When the parasitemia reached 18% in the 2nd calf, blood with 19% PCV was collected; erythrocytes were washed to remove plasma, and thin blood films were made on clean 75- by 25-mm microscope slides. The thin blood films were immediately force-air dried and fixed 30 minutes at −20 C in an absolute acetone (60%)-methanol (40%) mixture. The slides were stored at −70 C in sealed plastic bags containing anhydrous calcium chloride. The antigen slides were allowed to equilibrate to room temperature 1 hour before use. Similarly, normal, nonparasitized bovine blood was processed to serve as a negative control antigen.

Labeled Antibody—Commercially prepared liquid rabbit anti-bovine gamma globulin conjugated to fluorescein isothiocyanate[c] (RABGGFITC) was purchased and stored in multiple vials at −20 C. Titration of the conjugate resulted in a functional titer of 1:60.

Test Controls—Indirectly stained negative control serum failed to demonstrate fluorescence of the parasitic antigen at 1:20, although some nonspecific background fluorescence was occasionally present. For this reason, a base dilution of 1:40 was chosen for the test procedure. Indirectly stained positive control serum yielded maximal fluorescence at 1:60. Parasitic antigen slides directly stained with RABGGFITC (1:60) did not fluoresce. Normal nonparasitized bovine erythrocytic antigen stained with negative (1:40) and positive (1:60) serum, and post-stained with RABGGFITC (1:60) was negative. Parasitic antigen reacted with control positive serum (1:60) and indirectly stained with unlabeled rabbit anti-bovine gamma globulin (1:60), and then stained with RABGGFITC yielded little or no fluorescence.

Test Procedure—Standardized drops of negative (1:40) serum, positive (1:60) serum, and buffer control were directly applied to the surface of each antigen slide. Three test serums were placed on the same antigen slide, beginning at a 1:40 and extending to a 1:640 dilution in twofold increments. The test antigen slides were placed in a humid chamber and incubated 30 minutes at 37 C, following which they were washed 3 times with cold phosphate-buffered saline solution (pH 7.5) and forced-air dried. One standardized drop of RABGGFITC was directly placed onto the surface of the test sites, and the slide was incubated and washed as before, except with a final wash in cold distilled water. The unmounted slide was examined under oil immersion with a microscope[d] equipped with reflectance fluoroscopy apparatus, utilizing an Osram HBO-200 watt mercury vapor lamp, a (KP500) blue exciting filter, and a K515 suppression filter with 510-nm beam-splitting mirrors. All tests were performed in duplicate, and the final titer was interpreted at the highest maximum positive fluorescence. An attempt was not made to interpret degrees of minimal fluorescence.

Babesia bigemina parasites were observed on blood smears in each of the 5 cows an average of 7.6 ±0.5 days after exposure. These parasitemias were accompanied by a transient drop in packed cell volume.

[b] Azium, Schering Corporation, Dallas, Tx.
[c] Sylvana Company, Millburn, NJ.
[d] Leitz Ortholux Microscope, E. Leitz Inc, New York, NY.

Results

The results of tests conducted over a 175-day period on serums from 5 mature cows infected with *B bigemina* are presented (Table 1). The CF and IFA tests became positive an average of 10.2 days postinfection (PI). The measured average high titer for the CF test was 1:242 as compared with 1:735 for the IFA. The number of days required to reach these high titers varied only slightly and were not statistically significant. The average titer of all CF tests (60 in total) during the 70-day interval between PI days 14 and 84 was 1:41. The same serum tested with the IFA showed an average titer of 1:188, which was significantly higher ($P < 0.01$).

TABLE 1—Comparative Serologic Responses in Five Cattle Infected with *Babesia bigemina*

Item	Complement fixation	Indirect fluorescent antibody	Significance
Average days after infection that antibody response was detected	10.2 ± 1.8 days	10.2 ± 7.0 days	NS
Average recorded high titer	1:242	1:735	NS
Time at which high titer occurred	21.4 ± 8.0 days	17.6 ± 2.9 days	NS
Average titer during a 70-day period (day 14–84)	1:41	1:188	$P < 0.01$
Positive reactions between PI days 14 and 84	57/60 (95%)	57/57 (100%)	....
Positive reactions between PI days 95 and 175	24/60 (40%)	53/56 (95%)	....

PI = Postinfection. NS = Not significant.

During this same 70-day interval, 60 CF tests on serums from 5 cattle showed 57, or 95%, to be positive for *B bigemina* which was defined to be at least a 3+ reaction (no more than 25% hemolysis) at the 1:5 serum dilution. During the same interval, 57 tests with IFA showed 57 (100%) to be positive at a 1:40 or higher serum dilution.

Of 60 CF tests conducted on serum samples taken between PI days 98 and 175, only 24, or 40%, were positive. Only 1 of the 5 cattle remained consistently CF positive during this time period. During the same interval and on the same serums, 53 of 56 tests (95%) were positive, using the IFA test.

The average decreases in serum titers occurring with the CF and IFA tests are shown (Fig 1). Calculations were conducted on the log of the reciprocals for the 70-day interval (days 14–84) following artificial infection, as described by Snedecor.[9] Significant regression coefficients were detected when titers, expressed as the log of the reciprocals, were plotted against time. The average IFA regression coefficient was −0.0129, and the average CF regression coefficient was −0.0179. While the CF titers dropped slightly faster than the IFA titers during this time interval, the differences between the regression coefficients were not significant.[9] Plotted on the basis of this time interval, the CF would have reached the limits of its sensitivity (a 1:5 titer) in 94 days. This was generally confirmed by observations between PI days 98 and 175 when only 24, or 40%, were positive. Plotted on the same basis, the IFA theoretically would reach the limitation of its sensitivity at about 100 days. This did not prove to be the case. Serum titers in the IFA test virtually ceased to decrease as a function of time after day 98. In contrast, the titers remained fairly constant during this interval, with an average titer of 1:62. A regression coefficient of −0.00242 was calculated, but proved to be insignificant (Fig 1). The 5 cattle remained positive to the IFA tests, even though 3 negative responses occurred, during this period.

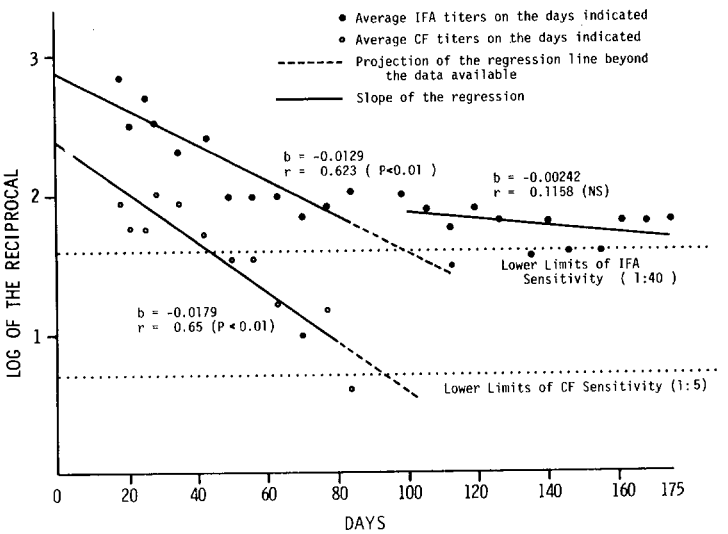

Fig 1—Comparative decrease in IFA and CF serum titers on postinfection days 18 to 84 and on 98 to 175 days (IFA only).

Discussion

This study confirms previous findings that CF antibodies do drop below a reliable limit fairly soon after infection occurs. During the first 84 days, regressions of both CF and IFA were similar. Because of the higher titers needed to recognize *Babesia* antibodies in the IFA test, little difference could be detected between the 2 tests during this interval. The leveling off of the IFA antibody titers after 90 days suggests the presence of different and more persistent antibodies than those associated with the CF response. In the presence of constant tick reinfestation, it is possible that CF antibodies are being continually stimulated, so that in practice the CF test may give more persistent reactions, but these results clearly indicate that the IFA is the more sensitive of the 2 procedures, in artificially infected cattle.

Serologic identification should be used in making the diagnosis of babesiasis, because the disease can be confused with anaplasmosis. Should *Babesia*-infected ticks, which occur in Mexico, be introduced into Texas and persist a short time before detection, severe death-losses among susceptible cattle might occur. A prompt serologic diagnosis would be most helpful for instituting effective chemotherapeutic control.

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Correction: Cardiovascular and Renal Functions in Normal Rhesus Macaques

In the report by C. T. Liu, appearing in AJVR, August, 1976, pages 969–974, the following author's change is made: Page 970, Table 3, column "Equation," last line, the entry is corrected to read: RU (mg/min) = TRBF · (A − RV difference (mg/ml)).