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9. ABSTRACT

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In cattle intentionally exposed to B bigemina, the BCT detected agglutinating antibodies simultaneously with the onset of first parasitemia. This reaction was observed to persist as long as 3 months, or long after the disappearance of parasitemia. Because of its simplicity and apparent specificity, the BCT may have use as a field test to aid in the diagnosis of B bigemina infections. The BCT results showed 100% agreement with the complement-fixation (CF) test on those serums prepared from blood collected within 3 months of infection.

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A Babesiasis Card Agglutination Test

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SUMMARY

A babesiasis card agglutination test (BCT) has been developed for detecting specific antibodies in cattle infected with *Babesia bigemina*. The agglutinating antigen was isolated from the blood of a splenectomized calf having 22% *B bigemina* parasitemia. The antigen was preserved with 0.02% formalin and stained with fast green dye. The BCT was performed by adding 1 drop of antigen and 2 drops of plasma or serum on a card and mixing for 5 minutes by rotation. Agglutination was visible in instances of positive reactions immediately after rotation.

In cattle intentionally exposed to *B bigemina*, the BCT detected agglutinating antibodies simultaneously with the onset of first parasitemia. This reaction was observed to persist as long as 3 months, or long after the disappearance of parasitemia. Because of its simplicity and apparent specificity, the BCT may have use as a field test to aid in the diagnosis of *B bigemina* infections. The BCT results showed 100% agreement with the complement-fixation (CF) test on those serums prepared from blood collected within 3 months of infection.

Although bovine babesiasis has been recognized as a serious economic disease for more than 80 years,[2] a practical serologic diagnosis has proved difficult. Identification of the acutely infected cattle has not presented a problem because *Babesia* spp can be detected in Giemsa-stained blood films. The diagnosis of subclinical infection is more difficult to do, since demonstrable parasitemia does not always occur. For this reason, there have been numerous attempts toward the development of serodiagnostic techniques for bovine babesiasis.

In the last decade, several serologic tests have been developed for the detection of *Babesia* spp antibodies in cattle (Table 1). The CF reaction constituted one of the earlier tests for diagnosis of bovine babesiasis.[8,20,21,23,27,29] The gel precipitation (GP) test seems to be a useful serologic technique for antigenic characterization of *Babesia* spp; however, its practical application has not been demonstrated.[22,29,30] Agglutination reactions provide a sensitive means for detecting *Babesia* spp antibodies in various systems, such as indirect hemagglutination (IHA) and direct hemagglutination,[5,7,12] capillary agglutination (CA),[17] and recently, a slide agglutination (SA) test.[6] Immunofluorescent antibody (IFA) techniques have been used for serodiagnosis of bovine babesiasis.[3,4,9,11,14-19,32]

TABLE 1—Serodiagnostic Tests for Bovine Babesiasis

Author(s)	Serologic test	*Babesia* spp
Mahoney (1962)[20]	CF	*B bigemina, B argentina*
Curnow and Curnow (1967)[7]	IHA	*B argentina*
Danov and Kyurtov (1967)[8]	CF	*B bigemina*
Mahoney (1967)[23]	CF	*B bigemina, B argentina*
Ross and Lohr (1968)[25]	IFA	*B bigemina*
Zwart et al (1968)[32]	IFA	*B bigemina, B major*
Lohr and Ross (1969)[17]	CA	*B bigemina*
Stepanova et al (1969)[27]	CF	*B bigemina*
Ross and Lohr (1970)[26]	IFA	*B bigemina*
Goodger et al (1971)[12]	IHA	*B bigemina, B argentina*
Todorovic et al (1971)[29]	CF	*B bigemina, B argentina*
Brocklesby et al (1971)[3]	IFA	*B major*
Donnelly et al (1972)[9]	IFA	*B divergens*
Goldman et al (1972)[11]	IFA	*B bigemina, B berbera*
Joyner et al (1972)[15]	IFA	*B divergens, B major*
Burridge et al (1973)[4]	IFA	*B bigemina*
Curnow (1973)[6]	SA	*B bigemina*
Johnston et al (1973)[14]	IFA	*B argentina*

CF = Complement fixation; IHA = indirect hemagglutination; IFA = immunofluorescent antibody; CA = capillary agglutination; SA = slide agglutination.

These serologic tests showed a high degree of specificity for each *Babesia* spp parasite. Because of the different sensitivities of each technique, *Babesia* spp antibodies can be detected for various periods after infection, depending on the test used.[13,24] The present report describes a rapid card agglutination reaction and its application for the detection of *B bigemina* infection in cattle.

Materials and Methods

Antigen Preparation—The card antigen was prepared from blood having a high percentage of *B bigemina* parasitemia produced in a splenectomized calf after 2 serial passages. Calf 1 was infected by intravenously inoculating 2.5 ml of packed infected erythrocytes previously mixed with an equal volume of 4 *M* dimethylsulfoxide (DMSO) and stored at −196 C in liquid nitrogen. When the parasitemia reached 1% in calf 1, 500 ml of blood was intravenously transferred to calf 2, which had previously been treated with 0.5 mg of dexamethazone* per kilogram of body weight given each day for 5 days before blood transfer. Calf 2 developed a 22% *B bigemina* parasitemia with a packed cell volume (PCV) of 18% at 62 hours after exposure. At this time, 5 L of blood was collected in ethylenediaminetetraacetic acid (dipotassium salt)† (1.2 g/L) by cannulation of a carotid artery. The blood was stored overnight at 4 C before processing.

The plasma was separated by centrifugation in a refrigerated centrifuge at 1,085 × g for 20 minutes. The sedimented *B bigemina*-infected erythrocytes were then washed

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* Aslum, Schering Corporation, Bloomfield, NJ.

† Barker Chemical Company, Phillipsburg, NJ.

twice in a sterile isotonic saline solution (0.85% NaCl) and lysed with sterile 0.35% NaCl according to the method described by Mahoney.[22] After lysis, the lysate was centrifuged at 8,000 × g in a refrigerated centrifuge for 30 minutes. The supernatant was discarded, and the sediment was washed once in a sterile antibiotic saline solution (containing 10,000 units of penicillin and 10 mg of streptomycin per ml) and stored at 3 to 5 C. A thin smear of the sediment when stained by the Giemsa technique revealed concentrated *B bigemina* parasites.

To each milliliter of *B bigemina* parasitic suspension, 2 volumes of a sterile antibiotic saline solution was added. This suspension was centrifuged in a refrigerated centrifuge at 5,000 × g for 30 minutes; the resulting supernatant was discarded, and the sediment was resuspended in 5 volumes of sterile isotonic saline solution to which 0.025 ml of 1% fast green dye[c] was added and kept at 3 to 5 C for 2 hours with occasional agitation. Excess dye was removed by centrifuging this suspension and discarding the supernatant. The sediment was suspended in formolized saline solution (0.02%) in dilutions ranging from 1:2 to 1:64. Each antigen dilution was tested for agglutination reaction with 5 known positive and 5 known negative plasma or serum samples.

Test Performance—A technique similar to the anaplasmosis card test described by Amerault and Roby[1] was used for the BCT. A Brewer diagnostic card[d] was used. Two drops of plasma or serum being tested and 1 drop of antigen was placed on the card and agitated by rotation for 5 minutes at moderate speeds at room temperature. The agglutination reaction was read and recorded at 1+, 2+, 3+, or 4+, with 4+ representing strong agglutination and 1+, minimal response. Clumping or agglutination was not apparent in negative samples.

Plasma or serum samples tested were prepared from blood collected from cattle before or during infection with *B bigemina*. Additional normal samples were collected from cattle never exposed to *Babesia spp* or to other known hemotropic infections. Tests were also performed on samples collected from cattle infected with *Anaplasma marginale* and from splenectomized and intact calves infected with *B bigemina*.

Complement-Fixation Test—The CF antigen was prepared from the blood source used for the BCT antigen and test was done as previously described by Todorovic et al.[29]

[c] Allied Chemical Company, Morristown, NJ.
[d] Hynson, Westcott & Dunning, Inc, Baltimore, MD.

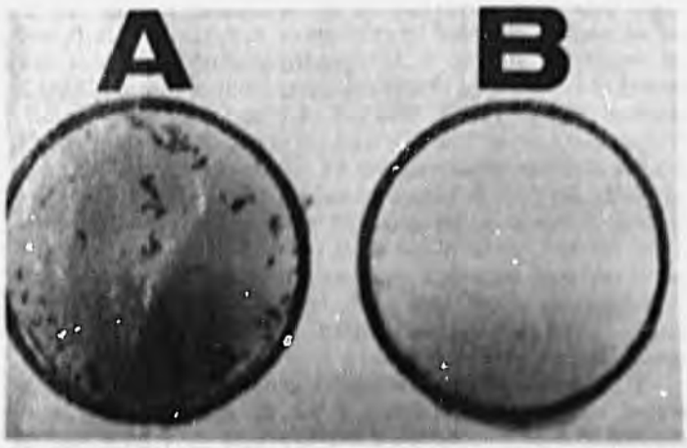

Fig 1—Positive (A) and negative (B) agglutination reactions observed in babesiasis card agglutination test for detecting *Babesia bigemina* antibodies.

Results

The pattern of agglutination reaction observed in the BCT is shown (Fig 1). The reaction was visible 2 to 3 minutes after rotation of the test mixture, but reached a maximal response in approximately 5 minutes. A photomicrograph of the *B bigemina*-infected blood used in preparation of the BCT antigen and the resulting antigen consisting of fast green dye-stained *B bigemina* parasites and parasitic fragments are illustrated (Fig 2 and 3). A 1:10 dilution of antigen seemed to give optimal agglutination reaction.

The relationship between anemia as measured by PCV, *B bigemina* parasitemia as determined on thin Giemsa-stained blood smears, and the BCT response of 7 adult cattle artificially infected with *B bigemina* is presented (Fig 4). The *B bigemina* parasites appeared in peripheral blood 5 days after exposure. Seemingly, the first detectable agglutinins correlated closely with

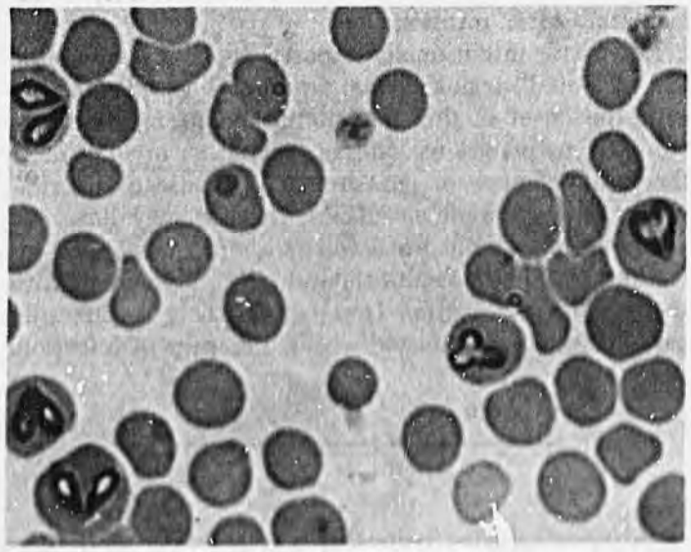

Fig 2—*Babesia bigemina*-infected blood for preparing concentrated parasite suspension. Giemsa stain; × 1,000.

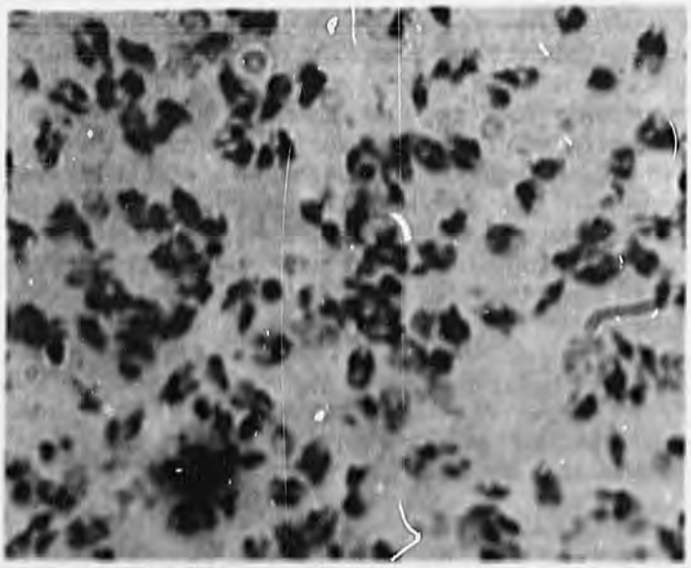

Fig 3—*Babesia bigemina* (erythrocyte-free) parasite suspension for preparation of agglutinating antigen for the babesiasis card agglutination test. Giemsa stain; × 1,000.

TABLE 2—Results of Babesiasis Card Agglutination (BCT) and CF Tests for Detection of *Babesia bigemina* Antibodies in 7 Adult Cattle, and Their Relationship with Parasitemia (P)

Days before and after infection	Cattle No. 299			903			904			493			497			498			361		
	BCT	CF	P(%)	BCT	CF	P(%)	BCT	CF	P(%)	BCT	CF	P(%)	BCT	CF	P(%)	BCT	CF	P(%)	BCT	CF	P(%)
−10	Neg	Neg	Neg	Neg	.....	Neg	Neg	.....	Neg	Neg	.....	Neg	Neg	.....	Neg	Neg	.....	Neg	Neg	.....	Neg
− 3	Neg	Neg	Neg	Neg	.....	Neg	Neg	.....	Neg	Neg	.....	Neg	Neg	.....	Neg	Neg	.....	Neg	Neg	.....	Neg
0	Neg	Neg	Neg	Neg	.....	Neg	Neg	.....	Neg	Neg	.....	Neg	Neg	.....	Neg	Neg	.....	Neg	Neg	.....	Neg
4	Neg	Neg	Neg	Neg	.....	Neg	Neg	.....	Neg	Neg	.....	Neg	Neg	.....	Neg	Neg	.....	Neg	Neg	.....	Neg
6	Neg	Neg	Neg	Neg	.....	Neg	Neg	.....	Neg	Neg	.....	0.1	Neg	.....	Neg	Neg	.....	0.1	Neg	.....	Neg
8	Pos	Pos	0.01	Neg	.....	Neg	Neg	.....	Neg	Pos	Pos	Neg	Pos	Pos	0.1	Pos	Pos	Neg	Pos	Pos	0.1
11	Pos	Pos	0.1	Pos	Pos	0.1	Pos	Pos	0.1	Pos	Pos	Neg	Pos	Pos	0.1	Pos	Pos	Neg	Pos	Pos	0.1
13	Pos	Pos	0.1	Pos	Pos	0.1	Pos	Pos	0.1	Pos	Pos	Neg	Pos	Pos	0.1	Pos	Pos	Neg	Pos	Pos	Neg
18	Pos	Pos	Neg	Pos	Pos	Neg	Pos	Pos	0.1	Pos	Pos	Neg	Pos	Pos	Neg	Pos	Pos	Neg	Pos	Pos	Neg
25	Pos	Pos	Neg	Pos	Pos	Neg	Pos	Pos	0.1	Pos	Pos	0.1	Pos	Pos	Neg	Pos	Pos	Neg	Pos	Pos	Neg
32	Pos	Pos	Neg	Pos	Pos	Neg	Pos	Pos	Neg	Pos	Pos	Neg	Pos	Pos	Neg	Pos	Pos	Neg	Pos	Pos	Neg
39	Pos	Pos	Neg	Pos	Pos	Neg	Pos	Pos	Neg	Pos	Pos	Neg	Pos	Pos	Neg	Pos	Pos	Neg	Pos	Pos	Neg
53	Pos	Pos	Neg	Pos	Pos	Neg	Pos	Pos	Neg	Pos	Pos	Neg	Pos	Pos	Neg	Pos	Pos	Neg	Pos	Pos	Neg
60	Pos	Pos	Neg	Pos	Pos	Neg	Pos	Pos	Neg	Pos	Pos	Neg	Pos	Pos	Neg	Pos	Pos	Neg	Pos	Pos	Neg

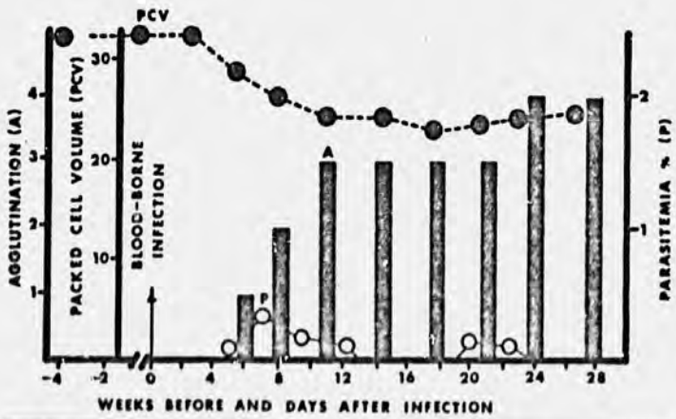

Fig 4—Relationship between anemia (packed cell volume), percentage of parasitemia with *B bigemina*, and appearance of agglutination reaction in the babesiasis card agglutination test of cattle infected with *B bigemina*.

the occurrence of demonstrable *B bigemina* parasitemia. The agglutination reaction persisted with increasing intensity, reaching a maximum of 4+ on postexposure (PE) day 24. The demonstrable *B bigemina* parasitemia was recorded from PE days 5 to 13 and from PE days 19 to 24. The lowest PCV values of 23% were recorded on PE day 18.

The BCT results compared favorably with those of the CF test on serum samples prepared from blood collected from cattle exposed to *B bigemina* infection (Table 2). For a 60-day period after exposure or an average of 52 days after the first evidence of *B bigemina* parasitemia, there was 100% agreement between the 2 tests.

The comparative results of using the BCT and the CF test on 289 serum samples from 13 *Babesia*-infected cattle, 58 samples from 58 *A marginale*-infected cattle, and 140 samples from 140 noninfected cattle are presented (Table 3). Reactions to BCT or CF test did not occur in the *A marginale*-infected and the noninfected cattle. Of the 289 samples from known *Babesia*-infected cattle, all had a positive response to the CF test, and 280 (97%) had a positive response to the BCT. The duration of the artificially induced *Babesia* infection in the 13 cattle tested did not exceed 4 to 5 months.

In addition, in plasma and serum samples prepared from blood collected from splenectomized calves infected with *B bigemina*, demonstrable agglutinins were first seen on PE day 20. In the intact calves, demonstrable agglutinins were first seen on PE day 13.

TABLE 3—Results of Testing Three Groups of Cattle with Babesiasis Card Agglutination and CF Tests

Group of samples	No. of cattle	Total No. of samples	No. of samples: CF test, Negative results	CF test, Positive results	BCT test, Negative results	BCT test, Positive results
Babesia bigemina-infected cattle	13	289	0	289	9	280
Anaplasma marginale-infected cattle	58	58	58	0	58	0
Noninfected cattle	140	140	140	0	140	0

The BCT antigen preserved at refrigeration temperature (3 to 5 C) was found reactive during 4 months of storage. Seemingly, reactivity decreases with time of storage, and may require more than 5 minutes of rotation to produce a visible agglutination reaction when it has been stored for long periods.

Discussion

Although several serodiagnostic tests for bovine babesiasis have been reported (Table 1) and noteworthy advances have been made in attempts to purify *Babesia* spp antigens from host substances by physical and chemical fractionation,[10] a simple practical diagnostic test has not yet been developed. Such a test should meet the following criteria: (1) test is simple to perform; (2) interpretation or results are free of subjectivity; (3) test is done rapidly; (4) cost is minimal; (5) test results are sufficiently sensitive and specific; and (6) test must be repeatable in various laboratories or under different field conditions. The BCT seems to meet most of these criteria and compares favorably with other available serologic methods, such as CF, IHA, IFA, and precipitation tests. Although the CA test observed by Lohr and Ross[11] is simple to perform, serum samples must be heat inactivated and test reactions cannot be read at once. Recently, Curnow[4] reported the SA test for the detection of, and for the

antigenic characterization of, *B bigemina* antibodies in serum of cattle in Australia and, like the BCT, it does not require special storage facilities and equipment for its performance. The BCT, by virtue of its simplicity and rapid action, is indicated for field work or routine laboratory screening procedures. Inactivated plasma or serum can be used, but is not necessary, for this determination. Plasma reactions generally appear stronger and more rapidly than those occurring with serum.

Even though the BCT antigen contains a large concentration of *B bigemina* parasites, erythrocytic contaminants probably occur. This was best evidenced by a few false positive reactions observed in cattle injected with a large volume of nonparasitized blood. A similar response has been reported in other hemotropic infections.[31]

Attempts to produce the BCT antigen from blood of cattle with low percentage of *B bigemina* parasitemia (less than 5%) were unsuccessful. It was estimated a total of 1,000 test units can be prepared from 5 L of blood having a *B bigemina* parasitemia of 22% and a PCV of 18%.

The BCT antigen tested in this investigation seems to have a considerable degree of specificity. According to Curnow,[6] the SA test is even strain specific. *Babesia bigemina* card antigen was unaffected by normal serums and antiserums against *A marginale*, whereas it reacted strongly in the presence of immune serums from homologous infection. In many areas of the world, considerable efforts are being made to develop effective premunization programs against bovine babesiasis.[28] There would be obvious advantages favoring a simple practical and portable diagnostic test to confirm the immune status of cattle or, in the quarantine zones, to ensure that no infected cattle enter clean areas. The BCT seems ideally suited for this type of operation, because the test could be carried out on plasma and readings made under field conditions. The BCT compared favorably with the CF test; however, the CF test has the disadvantage of complexity and the need for special laboratory equipment. The BCT is well suited for epizootiologic survey purposes. Large numbers of specimens can be readily tested at small expense.

Studies are underway to evaluate the strain specificity of this antigen more effectively, as well as to determine the persistence of agglutinating antibodies after an acute infection and the relationship of these reactions to carrier status.

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