AGENCY FOR INTERNATIONAL DEVELOPMENT
WASHINGTON, D. C. 20523

BIBLIOGRAPHIC INPUT SHEET

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0178

1. SUBJECT CLASSIFICATION

A. PRIMARY
Agriculture — AL72-0000-0000

B. SECONDARY
Pests of animals

2. TITLE AND SUBTITLE
Production of complement-fixation test antigen for serodiagnosis of Theileriasis in white-tailed deer (Odocoileus virginianus)

3. AUTHOR(S)
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4. DOCUMENT DATE	5. NUMBER OF PAGES	6. ARC NUMBER
1970	7p.	ARC

7. REFERENCE ORGANIZATION NAME AND ADDRESS
Tex.A&M

8. SUPPLEMENTARY NOTES (*Sponsoring Organization, Publishers, Availability*)
(In Am.j.of veterinary research,v.31,no.5,p.879-885)

9. ABSTRACT

10. CONTROL NUMBER
PN-RAA-127

11. PRICE OF DOCUMENT

12. DESCRIPTORS
Antigenic determinants
Complement fixation
Theileriasis
Deer

13. PROJECT NUMBER

14. CONTRACT NUMBER
CSD-1947 Res.

15. TYPE OF DOCUMENT

AID 590-1 (4-74)

Production of Complement-Fixation Test Antigen for Serodiagnosis of Theileriasis in White-Tailed Deer *(Odocoileus virginianus)*

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SUMMARY

Four complement-fixing (CF) antigens were produced from washed erythrocytes collected from a deer with a 73% theilerial parasitemia. For production of antigens, erythrocytes were lysed by ultrasonic disruption (method I, antigen 1), with distilled water (method II, antigen 2), with carbon dioxide-saturated water (method III, antigen 3) and by a mechanical tissue homogenizer (method IV, antigen 4). Production methods were compared on the basis of productivity, economy, adaptability for large-scale production, potency, anticomplementary activity, and specificity of antigens. Production methods in decreasing order of desirability were I, II, III, and IV. Method I yielded 46.77 test units of antigen per milliliter of packed erythrocytes, whereas methods II, III, and IV yielded 20.46, 8.77, and 2.77 test units, respectively.

Antigens 1, 2, and 3, in tests with the same reagents, were reactive with specific serum antibodies in deer infected with *Theileria* and were of satisfactory potency for routine diagnostic use. Antigen 4 had substandard potency and contained an excess of color that interfered with reading of CF test reactions. Specificity of the antigens was shown by their failure to react with serum from cattle and deer infected with *Anaplasma marginale*, normal bovine serum, serum of cattle infected with *Eperythrozoon wenyonii*, and serum from deer infected with *Babesia*.

Antigens were prepared by ultrasonic disruption of erythrocytes obtained from deer with theilerial parasitemias ranging between 0.1 and 80.0%. Blood of deer with high parasitemias yielded the most desirable antigens.

Attempts to detect circulating soluble antigens that could be used in CF and gel diffusion techniques for diagnosis of theileriasis in deer were unsuccessful.

Submitted for publication July 14, 1969.

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Based on a thesis submitted by the senior author to the Graduate College, Texas A&M University, College Station, in partial fulfillment of the requirements for the M.S. degree.

Two species of *Theileria* were reported in the United States, *Theileria mutans* in cattle[11] and *Theileria cervi* in white-tailed deer,[5,a] The presence of nonpathogenic *Theileria mutans* indicated that the United States may have the vectors that would permit the establishment of *Theileria parva* (cause of East Coast fever) if it were accidentally imported.

Diagnosis of theileriasis is one of the most important and difficult problems confronting those investigating the disease and is dependent upon the demonstration of schizonts or the erythrocytic parasites, or both, in blood and tissue smears. Differential diagnosis of the diseases caused by various species of *Theileria* is based upon the geographic distribution, degree of parasitemia, pathogenicity, epizootiologic features, signs, pathologic changes, nature of the immunity, and results of cross-immunity tests.[8]

Diagnosis of the carrier state is extremely difficult, because after initial infection, theileriasis in deer usually becomes an inapparent infection and organisms are difficult to demonstrate. Inoculation of blood from suspected cases into susceptible animals and splenectomy of premune animals have been used for diagnostic purposes.

The pleomorphic nature of the organism and the marked morphologic similarities of organisms within the family Theilerdae make recognition of specific *Theileria* difficult and create taxonomic problems.[6] The development of a system of serologic classification with cross-absorption techniques may result in better understanding of the relationships among these intraerythrocytic pathogens.[6]

A capillary agglutination test for detection of theilerial infection was developed by Schaeffler,[10,a] using antigen prepared by sonication of erythrocytes from white-tailed deer (*Dama virginiana*) infected with *Theileria cervi*. Kuttler *et al.*[6] prepared a CF antigen from deer blood with high theilerial parasitemia. Later Kuttler and Robinson[7] prepared an antigen from deer erythrocytes infected with *Theileria* for use in the capillary tube agglutination test. These workers reported that the capillary agglutination test, when compared with a CF test using an experimental antigen, was more sensitive and detected a higher percentage of theilerial infections. However, they stated that the CF test was of some value in detecting infections and that a refined CF antigen would be valuable in cross typing the organisms in theilerial infections.

The purpose in the present work was to determine whether an improved CF antigen could be developed which would be sufficiently sensitive to detect theilerial infections in carrier animals. To achieve such a goal, 4 methods of producing CF antigens were studied, using infective material collected from the same donor animal. Production methods were compared for ease of production, economy, and practical application. The antigens were compared for potency, specificity, and anticomplementary (AC) activity. The optimal stage of parasitemia in the deer for collection of blood for production of CF antigen was determined. In addition, attempts were made to detect the presence of circulating soluble antigens that would be of diagnostic value by the CF and gel diffusion techniques.

Materials and Methods

Antigen Production A healthy, splenectomized deer (No. 498) that had been raised in captivity was used for antigen production. Samples of serum from this deer were collected twice weekly for 18 months, tested by the CF and capillary tube agglutination tests (CAT), and were shown to be free of antibodies against *Theileria* and *Anaplasma marginale*. Stained blood smears were also examined twice weekly to ensure that the deer did not have other blood parasites. After 18 months' testing, the deer was inoculated intravenously with 10 ml of infective blood from a theilerial

a Schaeffler, W. J. *Theileria cervi* Infection in White-Tailed Deer (*Dama virginiana*) in the United States. (M.D. Diss., University of Illinois, Urbana, 1952.)

carrier deer (No. 477). Purity of the theilerial infection in the donor deer was determined by testing serum 2 times per week by the CF and CA tests and by microscopic examination of stained blood smears for more than one year.

Theilerial body counts and packed cell volume (PCV) determinations were made each day after deer 498 was inoculated. The animal was exsanguinated when the parasitemia reached 73.0% and the PCV was 20.0%. A liter of blood was collected in an equal volume of Alsever's solution. The erythrocytes were sedimented and washed 4 times in 0.85% sodium chloride solution by centrifugation at 1,060 g for 30 minutes. This yielded 156 ml. of packed erythrocytes which were divided into four 39-ml. portions. A given portion was processed by 1 of the following 4 antigen-production methods.

Method I.—The antigen was prepared by ultrasonic disruption and differential centrifugation of the packed cells, as described by Rogers *et al.*[9] This constituted antigen 1, which was stored at −60 C. until used.

Method II.—Antigen 2 was prepared according to the procedure described by Kuttler *et al.*[8] for the preparation of theilerial CF antigen. The antigen was stored at −60 C. until used.

Method III.—For production of antigen 3, packed cells were lysed by adding 30 volumes of cold (2 to 3 C.) carbon dioxide-saturated water and allowing to stand overnight at 4 C. Erythrocyte stroma was then treated and stored as in Method II.

Method IV.—Erythrocytes for antigen 4 were lysed by treatment in a tissue homogenizer[1] for 2 minutes at maximal speed. Erythrocyte stroma was treated and stored as in method II.

Evaluation of Antigen.—The 4 theilerial antigens prepared as described above were titrated against the standard theilerial antiserum to determine their potency and AC activity. Titrations were conducted according to the procedure described[4] for anaplasmosis antigen titration, except that deer serum was inactivated at 56 C. for 30 minutes. Standard positive serum used in the titrations was obtained from a known theilerial carrier deer (No. 477). Standard negative serum was obtained from deer (No. 130) which was shown for 2 years to be free from *Theileria* and other blood parasites as determined by 1-time per week testing of serum by CF and CA tests and by microscopic examination of stained blood smears. Standard serums were stored in 2-ml. portions at −25 C. until used. Guinea pig serum used as the source of complement in the CF procedures was obtained from adult animals by exsanguination. Serums were pooled, clarified by centrifugation, sealed in glass vials, and stored at −60 C. until used. Hemolysin was obtained.[4] The effect of freezing and thawing on the quality of the antigens was determined by titration of the antigens before and after freezing and thawing for 8 successive times.

Productivity comparisons were made based on the number of test units obtained from 1 ml. of packed erythrocytes. Test units were calculated according to the procedure outlined by Gates *et al.*[4] for comparing production methods of bovine anaplasmosis antigen. Test units per milliliter of packed red blood cells (RBC) were calculated by the following formula:

$$\text{Test units/ml. of RBC} = \frac{\text{ml. of antigen} \times \text{titer} \times \text{test units/ml. of antigen}}{\text{ml. of packed RBC}}$$

Efficacy of Complement-Fixing Antigen for Diagnostic Test.—Serums from 47 deer and 2 antelopes (*Antilocapra americana*) were tested by the diagnostic CF test.[8] Each serum was tested against antigens 1, 2, and 3. Antigen 4 had a low titer and was considered unsatisfactory for use in these tests. The serums were classed according to the stage of the theilerial infection in the deer at the time serum samples were obtained, thus (1) negative, no evidence of infection, (2) chronic or early infection, parasitemias of less than 2%, and (3) acute infections, parasitemias of 2% or greater.

Specificity of Antigens.—Serums from 2 deer with anaplasmosis, 2 deer infected with *Babesia*, 4 healthy calves, and 5 calves with anaplasmosis were tested by

[1] Vir-Tis Model 45 tissue homogenizer, the Vir-Tis Company, Inc., Gardiner, New York, N.Y.

[4] Anon. A Manual for Conducting the Complement Fixation Test for Anaplasmosis. Agricultural Research Service, U.S. Department of Agriculture, Aug. 1958.

[8] Agricultural Research Service, U.S. Department of Agriculture, Beltsville, Md.

TABLE 1—Hematologic Data on Blood That Was Used to Prepare Complement-Fixing Antigens from Deer with Varying Degrees of Theilerial Parasitemia

Deer No.	Parasitemia* (%)	Packed cell volume (%)
58	0.1	39
477	8.0	24
58	17.0	29
58	53.0	23
58	80.0	13

* % of erythrocytes with organisms, as determined by microscopic examination of Geimsa-stained blood smears.

the CF test, using antigens 1, 2, and 3. Serums for 2 calves infected with *Eperythrozoon wenyonii* were similarly tested.

Comparison of Antigen Titer with Parasitemia.—The method for preparing theilerial antigen 1 described above was employed to prepare antigens from blood of deer at different stages of parasitemia as shown in Table 1. These antigens were titrated as previously described.

Soluble Complement-Fixing and Precipitin Antigens. During the process of preparation of the theilerial CF antigens, a portion of the supernatant fluid from each centrifugation was tested for soluble CF and precipitin antigens. To determine CF activity, the supernatant fluids were titrated in the manner used to titrate the particulate antigens. Precipitating activity was tested by a simplified double diffusion agar precipitin technique described by Crowle.[7]

Results

Antigen 1 produced by ultrasonic disruption of infected erythrocytes had high antigenic strength (Table 2). Antigens 2 and 3 had moderate antigenic strength, but antigen 4 had low titer and contained an excess of color. Anticomplementary activity was not encountered in any of the 4 antigens.

Freezing and thawing of antigens for 8 successive times caused aggregation and flocculation of the antigenic material. Also titrations of the antigens revealed a slight decrease in titer. However, ultrasonic treatment of the final dilution of antigen 1 and homogenization of the other 3 antigens in the tissue homogenizer caused disruption of the aggregates and completely restored the original titer. The need for reprocessing stored antigen was eliminated by storage in small quantities that did not require repeated freezing and thawing between uses.

A comparison of the quantity of antigen produced by each method was based on the number of test units obtained from 1 ml. of packed erythrocytes, using the formula outlined above. Test units of antigens obtained by the 4 methods are shown (Table 2).

The results of CF tests on serum samples of 47 deer, using theilerial antigens 1, 2, and 3, are tabulated (Table 3). Antigen 4 was not included in these tests because it was not considered a suitable antigen. Of the CF tests on serums from 17 deer known to be free of *Theileria*, false positive test reactions did not occur with any of the antigens. In the results of tests using antigen 1, on the 15 deer with 2.0% or less theilerial parasitemias, 10 deer (66.7%) were test positive and 5 deer (33.3%) were suspect (10.0 to 75.0% fixation of com-

TABLE 2—Comparison of the Yield and Potency of Complement-Fixing Antigens Produced from Infected Erythrocytes by 4 Methods

Antigen No.	Cells used* (ml.)	Antigen yield**	Antigen titer†	Test units and ml. packed erythrocytes
1	[illegible]	[illegible]	[illegible]	[illegible]
2	[illegible]	[illegible]	[illegible]	[illegible]
3	[illegible]	[illegible]	[illegible]	[illegible]
4	[illegible]	[illegible]	[illegible]	[illegible]

* Volume of washed packed erythrocytes used. ** Number of milliliters of 5% [illegible] antigen suspensions obtained. † Reciprocal of highest dilution that marginally fixed 2 units of complement in presence of standard positive serum.

plement). In the CF tests on the same deer serums, using antigens 2 and 3, 8 deer (53.3%) were test positive, 6 (40.0%) were suspect, and 1 (6.7%) was test negative. When antigens 1 and 2 were used to test serums from 15 deer with more than 2% theilerial parasitemia all deer (100.0%) were test positive. When the same serums were tested against antigen 3, 13 (88.7%) were test positive and 2 (13.3%) were test suspect.

Serums from 2 antelopes were tested, using antigens 1, 2, and 3, for antibodies against *Theileria* at 13 days after the animals were inoculated with the organism. The serum of 1 antelope that was tested using antigen 1 gave a positive test reaction, and that of the other antelope gave a suspect test reaction. Both antelope serums in tests using antigens 2 and 3 gave suspect test reactions. Parasitemia was not detected in Giemsa-stained smears examined twice weekly.

Serums from the 11 calves (4 healthy calves, 5 with anaplasmosis as determined by the anaplasmosis CF test, and 2 infected with *E. ovinyonii*) were test negative in CF tests, using antigens 1, 2, and 3. Similarly, the 4 deer (2 with anaplasmosis as determined by the anaplasmosis CF test, and 2 infected with *Babesia*) were test negative in tests using antigens 1, 2, and 3.

The production time expressed in man hours for each 1 L. of blood processed by methods I, II, III, and IV were 3.5, 4.0, 6.0, and 3.5, respectively.

The number of antigenic test units produced had direct relationship to the parasitemia of blood used for antigen production (Table 4). Blood with the highest parasitemia gave the greatest number of test units and the most satisfactory antigen.

Attempts to demonstrate the presence of circulating soluble CF and precipitin antigens that could be of diagnostic value by the CF and gel-diffusion techniques were unsuccessful.

Discussion

The main objective in the present experiment was to develop techniques for preparing antigens used in CF tests for theilerial infections in deer. Four methods of producing CF antigens from parasitized erythrocytes taken from the same donor animal were compared for ease of production and practical application. The antigens were compared for potency, specificity, and AC activity.

Antigens 1, 2, and 3 were satisfactory for demonstrating circulating CF antibodies in deer with theileriasis. Antigen 1 was more sensitive than antigens 2 and 3 for detecting antibodies in chronic infections or early cases. Antigen 1 thus would be specifically useful for determining incidence and prevalence of theilerial infections in deer populations.

TABLE 3 Results of Complement Fixation Tests, Using Antigens 1, 2, and 3, of Serums Obtained from Deer Having Different Stages of Theileriasis

		Antigen 1			Antigen 2			Antigen 3		
Stage of theileriasis	No. of deer	Test positive	Suspect	Test negative	Test positive	Suspect	Test negative	Test positive	Suspect	Test negative
Free of theilerial infections	17	0	0	17	0	0	17	0	0	17
Percentage		0	0	100	0	0	100	0	0	100
Theilerial parasitemia of 2% or less	15	10	5	0	6	6	1	8	6	1
Percentage		66.7	33.3	0	40.0	40.0	6.7	53.3	40.0	6.7
Theilerial parasitemia of over 2%	15	15	0	0	15	0	0	13	2	0
Percentage		100	0	0	100	0	0	86.7	13.3	0

TABLE 4—Comparison Between Parasitemia Percentage and Antigen Titer

Deer No.	Parasitemia* (%)	Packed red blood cell volume (%)	Cells used (ml.)**	Antigen yield†	Antigen titer‡	Test units/ml. of packed erythrocytes
58	0.1	39	78	30.4	0	0
477	8.0	24	46	22.8	0.625	1.24
58	17.0	29	57	28.5	5.0	10.00
58	53.0	23	45	22.8	8 0	18.24
498	73.0	20	39	15.2	30.0	46.77
58	80.0	13	125	41.8	42.5	56.85

* % of erythrocytes with organisms, as determined by microscopic examination of Geimsa-stained blood smears. ** Total volume of washed packed erythrocytes used. † Number of milliliters of 5% (w/v) antigen suspension obtained. ‡ Reciprocal of highest dilution that completely fixed 2 units of complement in presence of standard positive serums.

Kuttler *et al.*[6] reported on a significant correlation between CF test titers of serums and the degree of parasitemia in acutely infected deer, using an experimental antigen comparable to antigen 2. In the present experiment, an antigen (antigen 1) was found to be more than twice as sensitive as antigen 2.

The theilerial antigens fixed complement only in the presence of serums from deer known to be infected with *Theileria*. False positive test reactions were not observed when the antigens were tested against normal bovine and cervine serums and serums from deer and cattle infected with other blood parasites. The specificity of the antigens for organisms within the genus *Theileria* remains to be determined. Anticomplementary activity was not observed in any of the 4 antigens. The absence of the AC activity which is often seen with hemoprotozoan CF antigens was attributable, at least in part, to immediate processing of erythrocytes after harvest.

Economy of production was determined for each method of antigen preparation based on man hours required for processing 1 L. of blood and on the number of test units obtained from 1 ml of blood. On the basis of this comparison, method I was the most economical and most productive. In addition this method could be easily adapted to large-scale production. Methods II and III were much less economical and productive than method I. In methods II and III, considerable amount of time was used to sediment the antigenic material from the large volumes of lysates. These methods could be adapted to large-scale production if a continuous flow system of centrifugation were available. Method IV was economical to do, but yielded an antigen of substandard potency. In addition, this antigen contained excessive color which interfered with interpretation of CF test reactions.

The relationship of percentage of the parasitized erythrocytes to strength of the antigen had been demonstrated by Franklin *et al.*[3] in large-scale production of anaplasmosis antigen. Data (Table 4) indicate that the idea of having maximum parasitism for antigen production is applicable for theilerial antigens from both the economical and the productivity points of view. Another conclusion that can be made is that the microscopically visible organisms are the primary source of the antigen.

The failure in the present experiment to demonstrate soluble antigens from serum that are reactive in the CF and gel precipitation tests does not prove that such antigens do not exist. Their demonstration may be possible if other methods of antigen production are used, such as the method for preparing soluble *Mycoplasma mycoides* antigen as described by Ancrenaz *et al.*[1] Possibly soluble antigens were not detected because the concentrations were not sufficient for detection in the tests used.

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