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


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.

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 and Theileria cervi in white-tailed deer. 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 theileriosis 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.

Diagnosis of the carrier state is extremely difficult, because after initial infection, theileriosis in deer usually becomes an inapparent infection and organisms are difficult to demonstrate. Induction 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 Theileridae make recognition of specific Theileria difficult and create taxonomic problems. The development of a system of serologic classification with cross-absorption techniques may result in better understanding of the relationships among these intraerythrocytic pathogens.

A capillary agglutination test for detection of theilerid infection was developed by Schaeffer, using antigen prepared by sonication of erythrocytes from white-tailed deer (Dama argus). Schaeffer, W. F. Theileria cervi infection in white-tailed deer. Thesis, University of Illinois, 1962

A capillary agglutination test for detection of theilerid infection was developed by Schaeffer, using antigen prepared by sonication of erythrocytes from white-tailed deer (Dama argus). Theileria mutans infected with Theileria cervi. Kutter et al. prepared a CF antigen from deer blood with high theilerial parasitemia. Later Kutter and Robinson 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, 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. Both the CF and capillary tube agglutination tests were used 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 were inoculated intravenously with 10 ml of infective blood from a theilerial

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 in an equal volume of Alsever's solution and washed 4 times in 0.85% sodium chloride solution by centrifugation at 1,600 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. This constituted antigen 1, which was stored at −60°C until used.

Method II. —Antigen 2 was prepared according to the procedure described by Kutte et al. 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 for 2 minutes at maximal speed. Erythrocyte stroma was treated and stored as in method II.

Evaluation of Antigens. —The theilerial antigens prepared as described above were titrated against standard theilerial antiserum to determine their potency and activity. Titrations were conducted according to the procedure described for anaplasmosis antigen titration, except that donor serum was activated at 50°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 sera 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. Sera were pooled, clarified by centrifugation, scaled in glass vials, and stored at −60°C until used. Hemolysin was obtained. 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. for comparing production methods of bovine anaplasmosis antigen. Test units per milliliter of packed red blood cells (PRBC) were calculated by the following formula:

\[
\text{Test units/ml of PRBC} = \frac{\text{ml of antigen} \times \text{titer} \times \text{test units/ml of antigen}}{\text{ml of packed PRBC}}
\]

Efficacy of Complement Fixing Antigen for Diagnostic Test. —Serums from 47 deer and 2 antelopes (Antilocapra americana) were tested by the diagnostic CF (test). 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 classified 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 7 deer with anaplasmosis, 2 deer infected with Babesia, 4 healthy calves, and 5 calves with anaplasmosis were tested by
TABLE 1—Hematologic Data on Blood That Was Used to Prepare Complement-Fixing Antigens from Deer with Varying Degrees of Theileria Parasitemia

<table>
<thead>
<tr>
<th>Deer No.</th>
<th>Parasitemia* (% of erythrocytes with organisms)</th>
<th>Packed cell volume (vol.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>58</td>
<td>0.1</td>
<td>20</td>
</tr>
<tr>
<td>477</td>
<td>8.0</td>
<td>24</td>
</tr>
<tr>
<td>66</td>
<td>17.0</td>
<td>20</td>
</tr>
<tr>
<td>58</td>
<td>53.0</td>
<td>23</td>
</tr>
<tr>
<td>58</td>
<td>80.0</td>
<td>13</td>
</tr>
</tbody>
</table>

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

The CF test, using antigens 1, 2, and 3, was similarly tested.

Comparison of Antigen Titer with Parasitemia. The method for preparing theilerial antigen 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 particular antigen. Precipitating activity was tested by a simplified double diffusion agar precipitin technique described by Crowle.

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 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 sera from 15 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 20% or less theilerial parasitemia, 10 deer (66.6%) were test positive and 5 deer (33.3%) were suspected 1000 to 75.0%, fixation of complement.
implement). 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. ewennyi) 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>
<thead>
<tr>
<th>TABLE 3 - Results of Complement Fixation Tests, Using Antigens 1, 2, and 3, of Serums Obtained from Deer Having Different Stages of Theileriosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage of Theileriosis</td>
</tr>
<tr>
<td>-----------------------</td>
</tr>
<tr>
<td>Free of Theilerial Infection</td>
</tr>
<tr>
<td>Theilerial parasitemia of 1% or less</td>
</tr>
<tr>
<td>Theilerial parasitemia of over 1%</td>
</tr>
</tbody>
</table>
unpublished known to cervine sera, from deer sera were tested against normal bovine sera and were not observed. What little antigen suspension obtained. 1 Reciprocal of highest dilution that completely fixed 2 units of complement in presence of standard positive sera.

Kuttler et al.\(^9\) reported on a significant correlation between CF test titers of sera 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 sera 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 sera and sera 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 hemoparasitic 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 labor hours required for processing 1 L of blood and on the number of titer units obtained from 1 mL of blood. On the basis of the 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 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 parasitemia 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 more equally viable organisms are the primary source of the antigen.

The failure in the present experiment to demonstrate soluble antigens from sera that are reactive in the CF test may be due to other factors, including the antigen preparation method used, the method for preparing soluble antigen, and the antigen concentration described by Aronski et al.\(^5\). Possibly soluble antigen was not detected because the concentrations were not sufficient for detection in the tests used.

### TABLE 4—Comparison Between Parasitemia Percentage and Antigen Titer

<table>
<thead>
<tr>
<th>Deer No.</th>
<th>Parasitemia* (%/mI.)</th>
<th>Packed red blood cell volume (%/mI.)</th>
<th>Cells used (ml)**</th>
<th>Antigen yield</th>
<th>Antigen titer (log$_{10}$)</th>
<th>Test titer (%/mI. of packed erythrocytes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>58</td>
<td>0.1</td>
<td>22</td>
<td>78</td>
<td>20.4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>477</td>
<td>8.0</td>
<td>24</td>
<td>46</td>
<td>22.8</td>
<td>0.025</td>
<td>1.24</td>
</tr>
<tr>
<td>58</td>
<td>17.0</td>
<td>20</td>
<td>57</td>
<td>28.5</td>
<td>5.0</td>
<td>10.00</td>
</tr>
<tr>
<td>58</td>
<td>23.0</td>
<td>23</td>
<td>45</td>
<td>22.8</td>
<td>8.0</td>
<td>15.24</td>
</tr>
<tr>
<td>122</td>
<td>73.0</td>
<td>20</td>
<td>39</td>
<td>15.2</td>
<td>30.0</td>
<td>40.77</td>
</tr>
<tr>
<td>58</td>
<td>80.0</td>
<td>13</td>
<td>125</td>
<td>41.8</td>
<td>42.5</td>
<td>56.95</td>
</tr>
</tbody>
</table>

* % of erythrocytes with organisms, as determined by microscopic examination of Giemsa-stained blood smears. ** Total volume of washed packed erythrocytes used. 1 Number of milliliters of 5% (w/v) antigen suspension obtained. 2 Reciprocal of highest dilution that completely fixed 2 units of complement in presence of standard positive sera.
References


