A COMPARISON OF 4 SEROLOGICAL TESTS IN THE DETECTION OF HUMORAL ANTIBODIES TO ANAPLASMOSIS IN CATTLE


Queensland Department of Primary Industries, Animal Health Station, Oonoonba, Queensland, 4810

SUMMARY: A capillary agglutination (CA), a complement fixation (CF), a plate agglutination (PT) and an indirect fluorescent antibody (IFA) test to detect humoral antibodies to Anaplasma marginale are described. Tests from 3, 4 or 5 groups of cattle were used to examine the efficiency of the tests.

Agreement between all 4 tests was 86.6%. Agreement between pairs of tests was greater. The CF test was the most sensitive while the PT test was the least sensitive. However the PT could be carried out very rapidly and was suggested as the best screening test, providing improved antigen preparation techniques could increase sensitivity.

The CA, CF and IFA tests all showed a stronger homologous antibody reaction when A. marginale antigen was tested against sera obtained from cattle infected with either A. marginale or A. centrale. Antibodies in the A. marginale sera were first detected by day 7 post-inoculation, rose to a peak around day 29 and were still present on day 200. Antibodies in the A. centrale sera were first detected by day 29 and rose to a peak around day 50 and had disappeared by day 150.

Introduction

A number of serological tests have been used to detect humoral antibodies to Anaplasma marginale infection in cattle. These include complement fixation (CF) (Rees and Mohler 1934), capillary agglutination (CA) (Ristic 1962), indirect immunofluorescence (IFA) (Schindler et al 1966) and card or plate agglutination (PT) (Amerault and Roby 1968). Much work has been conducted in comparing these tests in efforts to produce a simple, sensitive and effective serological aid to diagnosis. However, most of this work has been carried out using pairs of tests. Thus comparisons have been made between the CF and CA tests (Kuttler 1963; Jakat et al 1966; Hibbs et al 1966), the CF and PT tests (Amerault et al 1969, 1972; Christenberry and Alley 1972), and the CA and IFA tests (Ross and Lohr 1970; Lohr et al 1973).

In Australia Rogers (1971) has used the CA and CF tests to detect A. marginale antibodies in cattle. He reported only 68.3% agreement between the two tests from field sera although very good agreement occurred when experimental sera were used.

It seemed useful therefore to compare all 4 of the above serological tests for sensitivity, simplicity and specificity in detection of A. marginale infections in cattle in the Australian environment. A study on the use of dried blood collected on filter paper for use in the IFA similar to that described in Bailey et al (1967), was also conducted.

Differentiation between infections of A. marginale and A. centrale may be necessary in herds vaccinated with the latter. The serological response in groups of cattle infected with A. marginale and A. centrale, respectively was followed by the CA, CF and IFA tests using A. marginale antigen.

Materials and Methods

Anaplasma Organisms

An isolate of A. marginale, collected from a field outbreak at Cloncurry, Queensland, was inoculated into a splenectomised calf and the resulting parasitaemia frozen as a stabitate (Lumsden and Hardy 1965) by the method of Dalgleish and Mellors (1974) and designated Oonoonba-I. The calf was dosed with Primaquine* (2mg/kg) on days 8, 9 and 10 postinoculation to eliminate any circulating Theileria mutans. No other tick fever organisms were observed. Derivatives from Oonoonba-I in splenectomised calves were used for antigen production. The passage time in calves varied from 14 to 17 days.

Antigen Production

Antigens for CF, CA and PT tests were prepared in a similar manner. Blood was removed into 5% sodium citrate from an infected splenectomised calf whose parasitaemia was between 60 and 70% with a packed cell volume (PCV) of at least 20%. The blood was then washed 3 times in an equal volume of phosphate buffered saline (PBS) pH 7.2. To 100 ml aliquots of washed packed erythrocytes was added 100 ml of PBS. The cells in 200 ml volumes were then exposed to sonic vibration (50 watts using ½" disruptor horn of the sonicator) for 5 minutes. The released anaplasma and erythrocyte debris were concentrated by centrifugation at 9000 g for 30 minutes, the supernatant fluid then being discarded. The parasitic concentrate was then subjected to 10 minute periods in a 15 ml Pyrex tissue grinder. 3 second bursts of sonic vibration followed by centrifugation until visual remnants of erythrocyte debris were no longer detectable.

* IC, Australia Ltd, Melbourne
† B-I2 Sonifier, Branson Sonic Power Co., United States of America.
Antigen for the PT was stained with Giemsa. To a volume of the concentrated anaplasma, an equal volume of 20% Giemsa, buffered at pH 7.2 was added. After one hour, the mixture was centrifuged at 9000 g for 30 minutes, the supernate was discarded and the antigen reconstituted in an equal volume of PBS.

Antigen for the IFA was prepared as follows: 1500 ml of *A. marginale* infected blood containing approximately 20% infected cells from a splenectomised calf was inoculated into a second splenectomised calf. After 2 days, when 5-10% erythrocytes contained anaplasma, blood was removed directly into PBS in a ratio of 1 vol : 9 vols. The erythrocytes were then washed 3 times in PBS and then made up to their original concentration with PBS. Thin blood films were then prepared, air dried and covered with masking tape by the method of Parker (1971). The slides were then stored in boxes at −20°C.

Serological Tests

**Capillary agglutination** — A similar procedure for the standardisation of antigen and performance of the CA test to that described by Rogers (1971) was used, except that a result was recorded only as positive or negative and was read after 24 hours. Screening tests were carried out using whole serums, and titrated as described by Teakle (1966). A microtest similar to that described by Martin and Richie (1973) was used with the following differences. Commercial guinea pig complement* was titrated, in the absence of antigen; 2.5 haemolytic units were used for the test. The optimum dilution of antigen was calculated by using doubling dilutions from 1/5 against known positive and negative serums at 1/5. The highest dilution of antigen giving complete fixation was considered to have one unit of antigen. Three units of antigen were used in the test. Serum dilutions of 1/4 with a 1/2 serum control were set up for screening tests. A positive serum of known titre and also a negative serum were included as controls. Those serums showing more than 50% non-haemolysed cells at 1/4 were considered positive. Any fixation of complement below this level was regarded as negative. Serum were tested to titre using doubling dilutions from 1/4.

**Plate agglutination** — Batches of antigen were standardised by testing 2 positive serums to titre and the antigen either concentrated or diluted until the desired titre was achieved. 0.03 ml antigen was added to 0.03 ml test plate on a plate attached to a rocker. After 10 minutes of rocking the test was read as either negative or positive according to the presence of clumps of parasites.

**Complement fixation** — Cohn’s diluent, sheep red cells stored in modified Alsevers solution and haemolysin were used and titrated as described by Teakle (1966). A microtest similar to that described by Martin and Richie (1973) was used with the following differences. Commercial guinea pig complement* was titrated, in the absence of antigen; 2.5 haemolytic units were used for the test. The optimum dilution of antigen was calculated by using doubling dilutions from 1/5 against known positive and negative serums at 1/5. The highest dilution of antigen giving complete fixation was considered to have one unit of antigen. Three units of antigen were used in the test. Serum dilutions of 1/4 with a 1/2 serum control were set up for screening tests. A positive serum of known titre and also a negative serum were included as controls. Those serums showing more than 50% non-haemolysed cells at 1/4 were considered positive. Any fixation of complement below this level was regarded as negative. Serum were tested to titre using doubling dilutions from 1/4.

**Indirect immunofluorescence** — A test similar to that described by Johnston et al. (1973a) was used. Differences included the use of nail varnish circles to separate sera on the same slide, and of *A. marginale* antigen. The optics used were based on the optimum system for transmitted light described by Johnston et al. (1973b). A scoring system of degree of fluorescence from 0 to 4 plus was used. Serum were screened at 1/64 and were scored positive if they gave a 2 to 4 plus reading. Serum were tested to titre using doubling dilutions from 1/64.

**Sera**

Five groups of animals were used for collection of serums to evaluate the tests.

Group 1 comprised 19 animals (9 Herefords and 10 Brahman-Shorthorn crosses) which were experimentally infected with approximately 1010 *A. marginale* infected erythrocytes given intravenously (iv). Serums were obtained regularly over the period 7 to 220 days after inoculation in the Herefords and 7 to 60 days after inoculation in the Brahman crosses. A total of 202 serums were collected from the 19 animals.

Group 2 comprised 286 serums collected from individual animals on properties in areas free of the tick *Boophilus microplus*.

Group 3 comprised 531 serums collected from individual animals on holdings which were infected with *B. microplus*.

Group 4 comprised 5 Shorthorns, aged 17 to 20 months which were experimentally infected with approximately 107 *A. centrale* infected erythrocytes intravenously. Serums were obtained regularly over the period of 220 days after inoculation. A total of 60 serums were collected from the 5 animals.

All serums were stored without preservative at −20°C. Serums from animals in groups 1 and 4 were tested to titre by the CA, CF and IFA tests. Serums from the animals in groups 1, 2 and 3 were screened by all 4 tests.

Group 5 comprised 50 dried blood samples collected on Whatman No. 2 filter paper from 50 animals known to be anaplasmosis free and 100 samples from the animals of group 1. These samples were placed at room temperature for 5 days and than were stored in sealed envelopes at −20°C for one month. After this time, they were tested by IFA. Circular discs of volume 0.8 mm were cut and deposited on drops of 0.03 ml PBS which were in the nail varnish circles on the antigen slides. This gave an approximate serum dilution of 1/60. After one hour the discs were washed off and the IFA test proceeded.

**Results**

Comparison of the 4 serological tests in the detection of *A. marginale* infections in the serums of groups 1, 2 and 3:

The results of the CA, PT, CF and IFA tests, used as screens to detect the presence of *A. marginale* antibodies in the serums of groups 1, 2 and 3 are shown in table 1. No positive reactions were recorded by any of the tests in the known negative serums (group 2). All tests detected a large number of positive serums in both groups 1 and 3. The overall agreement between all 4 tests was 86.6% (both positives and negatives). Percentage agreement between pairs of tests was greater, being 91.6% and 88.3% between the IFA and CF, and the CA and CF respectively.

All tests were 100% accurate in the detection of the negative serums of group 2 and between 97 to 100% accurate in the detection of positive serums obtained 21 or more days after inoculation in group 1. The percentage of serums in group 3 which were detected as positive by the PT, CA, IFA and CF tests were 51.2, 52.7, 60.3 and 61.2% respectively.

Comparison of serological responses of group 1 (infected with *A. marginale*) and group 4 (infected with *A. centrale*):

The strength of humoral antibody response to *A. marginale* antigen in the cattle of groups 1 and 4 as detected by the CA, CF and IFA tests is shown in Figure 1. All tests showed a much stronger and longer response to the homologous antibody-antigen system (group 1 serums). Antibodies to *A. marginale* were detected by all 3 tests by day 7 postinoculation, rose to a peak around day 29 and then steadily declined but remained at detectable levels until at least day 200.

* Burroughs Wellcome, United Kingdom.
postinfection in some of the group 1 serums. *A. marginale* parasites were detected over the period 10 to 24 days postinoculation. The average titres for each test were on day 7 postinoculation IFA 1/71.5, CF 1/19.4, CA negative; on day 29 IFA 1/5210, CF 1/475.6, CA 1/60.5; and on day 200 IFA 1/84, CF 1/65.6, CA 1/0.8.

Antibodies in group 4 serums were not detected until between days 29 and 36 postinoculation, rose to a peak around day 50 and by day 115 only complement-fixing antibodies were present. The latter antibodies were detected up to day 150. *A. centrale* parasites were detected over the period 26 to 47 days postinoculation. The mean titres for each test were on day 36 postinoculation IFA 1/179.2, CF 1/128, CA 1/0.6; and on day 50 IFA 1/192.0, CF 1/44.8, CA 1/0.8. On day 115, only the CF test gave a mean titre (1/9.6).

Analysis of the filter paper IFA on the 150 dried blood samples (group 5).

The filter paper IFA clearly differentiated between the 50 known negative and the 100 known positive samples. All negative samples gave a fluorescence reading of 0, while all positive samples gave a reading of 2 or more. 82% of the positive discs gave the same fluorescence reading as the 1/64 dilution of serums obtained at the same time. The remaining 18% gave a weaker fluorescence reading than their corresponding serums; nevertheless they remained in the positive range.

**Discussion**

The main uses of serological tests as aids in the diagnosis of anaplasmosis are to delineate the disease and to ascertain the requirement for vaccination. Eradication, as is carried out in some parts of the United States of America (Franklin *et al* 1966), is not yet a practical possibility in Australia.

All 4 tests showed a high degree of specificity. These findings agree with those of previous workers (Ristic 1962; Kuttler 1963; Amerault *et al* 1972). The overall agreement between pairs of tests was lower than that reported by some other workers (Kuttler 1963; Amerault *et al* 1962). Also, percentage agreement became lower as more tests were compared.

The use of one test which can be carried out rapidly is thought to be of more value than a combination. The plate test fulfills this requirement. It gave the lowest accuracy of the 4 tests studied; however a result can be obtained in 10 minutes and the accuracy is within acceptable limits for a herd test. The effectiveness of the test would probably improve with more sophisticated methods.

**TABLE 1**

Comparisons of the CA, PT, CF and IFA Tests, used as Screens, to Detect the Presence of *A. marginale* Antibodies in the Serums of Groups 1, 2 and 3. The Overall Agreement between all Four Tests (both Positives and Negatives) in the 1019 Serums was 86.6%.

<table>
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<th>IFA AC Positive</th>
<th>Negative</th>
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<th>Negative</th>
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of antigen preparation. The test is used widely in the United States of America (Amerault et al 1969; Amerault and Roby 1971; Christenberry and Alley 1972) where it appears to be sensitive and shows very high correlation with the CF test.

The CF test was the most efficient test at detecting serological reactors. Also, it was the most sensitive test. The IFA has been shown to be very sensitive in other applications (Burridge 1971). However, emphasis in the IFA test used here was placed on producing a test which was not oversensitive. Hence a low magnification of X 200 and a transmitted light source of low wattage combined to give a relatively intensive test. This facilitated the differentiation between positives and negatives. The CF and IFA tests could be used as research tools in specific circumstances, for example to titre sera. A titre may be required to estimate the stage of infection or to attempt to differentiate between A. marginale and A. centrale.

Differences in antibody titre in the sera of animals infected with A. marginale or A. centrale were observed when tested against A. marginale antigen. The demonstration of titres in animals infected with A. centrale suggests that vaccinated herds should be avoided in serological surveys. Serums would have to be tested against both antigens if identification of the infecting parasite is required (Kuttler 1967).

The value of dried blood on filter paper as a source of antibody to aid in the diagnosis of haemoprotezoan infections in animals using the IFA technique has been demonstrated by Wilson (1969), and Kimber and Burridge (1972). From the small study carried out in these experiments, antibodies to A. marginale can be detected in this manner. The test has been used extensively in Africa (Anon 1972) in areas where communications have been poor and where the transportation of samples through the post has been a great advantage. Throughout most of northern Australia, transportation is good but distances are great and in some instances this modification of the IFA may be useful. The samples did not deteriorate after 5 days at room temperature.

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References


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