AN INDIRECT HAEMAGGLUTINATION TEST FOR THE DIAGNOSIS OF *BABESIA ARGENTINA* INFECTION IN CATTLE

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Introduction

The complement fixation (CF) test described by Mahoney (1962) has been used since 1964 for the control of *Babesia argentina* infection in cattle in north-eastern New South Wales. Approximately 0.5% of cattle tested have shown a positive CF reaction but have been negative to a transmission test. This is up to ten times the number of infected animals in some areas. Therefore it has been necessary to check all CF reactors by transmission test before placing holdings under quarantine. This is time consuming and expensive, but to quarantine holdings for 12 months or more on the basis of a positive CF test alone would mean the imposition of unnessary restrictions on many properties. There is, therefore, a need for another test capable of eliminating false-positive reactors to the CF test.

The indirect haemagglutination (IH) test was selected for trial. Boyden (1951) and Stavitsky (1954) described the absorption of various proteins onto tannic acid-treated erythrocytes and subsequent agglutination of such erythrocytes by specific antiserums. This method has been used subsequently for the diagnosis of protozoal diseases such as toxoplasmosis (Jacobs and Lunde 1957; Lewis and Kessel 1961; Chordi *et al* 1964) and amoebiasis (Lewis and Kessel 1961).

Materials and Methods

Complement Fixation Test

The complement fixation test was performed by the method suggested by Mahoney (1962) with slight modification. Red cell suspensions were standardised colorimetrically and in complement titrations the 50% endpoint (1 unit) was determined in a similar manner. In the test 3.3 such units were used.

Antigens for the CF test were prepared from bovine blood with between 25% and 40% of the erythrocytes containing *B. argentina* parasites. Stained blood films were examined to ensure that such blood was free of other blood protozoa.

The erythrocytes were lysed with cold (5°C) distilled water, and the mixture of parasites and erythrocyte stroma was prepared by the method described by Mahoney (1962). This mixture was then frozen rapidly to −79°C in 2 ml amounts and stored at this temperature until used. Preserved in this way it retains its antigenicity for at least six months. On the day of the test the mixture was thawed rapidly by immersing the ampoules in a waterbath at 37°C.

Antigens were titrated against a group of ten positive and six negative reference serums. These serums were from animals that had been subjected to transmission tests at the time the samples were collected. The positive samples were therefore from known infected animals and were selected so as to give as wide a variety of reactions as possible. Six of these samples were from blood-induced infections, serums being taken at 7 days and 1, 3, 6, 12 and 22 months after infection. The remaining four were from field infections, two being strongly positive (CF titres 1/320 and 1/80) the others weakly so (CF titres 1/10). These latter weakly positive serums were from animals whose recipient calves on transmission took 14 and 20 days respectively to react.

The appropriate dilution of antigen for use in the test was selected by using twice the amount in the highest dilution giving complete or near complete (++++ or +++) fixation with the weakest positive reference serum diluted 1/5. The usual antigen dilution used was between 1/8 and 1/16.

Bovine erythrocyte stroma antigen (BES) was prepared and stored in the same manner as the *B. argentina* antigen, except that uninfected bovine erythrocytes from several animals were used after pooling.

All reference serums and those positive to the CF test were also absorbed with BES then retested. An equal volume of undiluted BES was added to the serum and then allowed to stand at room temperature for two hours. After dilution, the particulate matter was removed by centrifugation.

Interpretation of CF Test. — A titre of 1/10 or more was considered to be positive. Occasionally infected animals show a positive CF titre that can be reduced by absorption with BES. Therefore, if after absorption a titre of 1/10 was still obtained, but a reduction in titre of more than one dilution occurred, the reaction was classed as "positive, partially absorbed by BES". If after absorption a titre of less than 1/10 was obtained the reaction was classed as "negative, completely absorbed by BES".

Indirect Haemagglutination Test

Analytical grade reagents were used throughout.

Phosphate buffered salines (PBS) of pH 7.2 and 6.4 were prepared by the formulae given by Daniel *et al* (1963).

Bovine albumin was obtained as a 30% solution from Commonwealth Serum Laboratories, Parkville, Victoria.

Normal rabbit serum was prepared from non-immunised domestic rabbits and absorbed with an equal volume of packed fresh sheep erythrocytes for two

hours at room temperature. It was heated at 56°C for 30 minutes and stored at −20°C until used.

Formalinised sheep erythrocytes were used in the test rather than fresh cells. Blood was obtained from a single animal, and the washed cells were treated with formalin by the method suggested by Weinbach (1958) and modified by Butler (1963). The cells were stored at 5°C after the addition of thiomersal to a concentration of 0.01%.

Tannic Acid-treatment of Cells.— The formalinised cells were washed once in isotonic saline (0.85%) and resuspended in saline to a 2.5% suspension (by volume). To one volume of cell suspension an equal amount of 1/2,000 tannic acid in isotonic saline was added. The mixture was incubated at 37°C for 20 minutes after which the cells were washed in one volume of PBS at pH 7.2.

Sensitisation of Tannic Acid-treated Formalinised Cells.— The cells were suspended in five volumes of PBS pH 6.4 and one volume of the appropriate antigen dilution (see below) added. The mixture was allowed to stand at room temperature for 30 minutes then centrifuged and the cells washed once in one volume of isotonic saline containing 1/200 normal rabbit serum. The sensitised cells were then resuspended as a 2.5% suspension in this diluent.

Antigen for the IH Test.— To prepare the IH test antigen the CF test antigen was added to an equal volume of cold (5°C) distilled water on the day of the test. This was then centrifuged at 27,000 *g* for 30 minutes in a Servall RC2 refrigerated centrifuge, the supernatant fluid being used as the antigen.

To increase the yield of the IH test antigen the sediment after this initial extraction could be refrozen to −79°C, thawed and extracted with distilled water in the same manner, several times if necessary. Each extract however was titrated separately before pooling and used only if found to be suitable (see below).

To determine the dilution of the antigen to use, portions of cells were sensitised with the undiluted antigen and antigen diluted 1/2, 1/4, 1/8 *etc.* The same group of positive and negative reference serums used as reference serums for the CF test were tested with each portion of sensitised cells. The antigen dilution selected for sensitising the cells for the test was the highest at which all positive reference serums showed a ++ reaction when diluted at 1/80. Antigen pools or extracts were rejected if at this dilution any of the negative reference serums showed a ++ or greater reaction when diluted at 1/20. In many cases antigens were used undiluted. Altogether 48 batches of CF antigen were tested and all gave satisfactory IH antigens at the initial extraction. Commonly the additional extractions were rejected however.

IH test antigens were used on the day of preparation to sensitise cells, and the sensitised cells were also used immediately. Whenever possible several batches of CF antigen were pooled, and all serums were tested using the same antigen or antigen pool for both CF and IH tests.

The BES antigen for the IH-inhibition test was prepared from the BES antigen used in the CF test. The method used was the same as for the *B. argentina* antigen except that only one extraction was made.

All serums were stored at −20°C without preservative.

The Test.— The test was carried out in perspex trays. On the day of testing all serums were absorbed with an equal volume of packed fresh sheep erythrocytes before dilution. Dilutions were made in PBS pH 7.2 containing 0.1% bovine albumin. The initial serum dilution was 1/20 and doubling dilutions 1/40, 1/80, 1/160 *etc* were prepared from it.

To each 0.5 ml of serum 0.05 ml of sensitised cells was added, the plates shaken vigorously immediately, and again after 10 minutes. They were then allowed to stand undisturbed for 3 hours at room temperature at which time settling patterns were distinct.

The group of reference serums used previously was included with each day's testing. Controls were included to determine if autoagglutination of the sensitised and non-sensitised cells occurred in the PBS diluting solution. Controls were included with each serum sample under test to see if it agglutinated formalinised or tannic acid-treated formalinised cells. The cells for the latter control were also subjected to PBS pH 6.4 for 30 minutes.

Reactions were graded according to the method described by Jacobs and Lunde (1957). The endpoint was taken as the highest dilution showing a ++ reaction.

Haemagglutination inhibition.— To check the specificity of each reaction BES and *B. argentina* IH test antigens were mixed separately with the serum before testing. This was also done with the reference serums each day.

One volume of serum was mixed with four volumes of the inhibiting antigen (used undiluted). This was then allowed to stand at room temperature for 2 hours after which any particulate matter was removed by centrifugation. The serum was then adjusted to a 1/20 dilution containing 0.1% bovine albumin.

Interpretation of the IH test.— A reaction to the IH test was considered to be positive if the reaction after inhibition with BES was at least three dilutions greater than after inhibition with *B. argentina* antigen, provided the titre after inhibition with BES was at least 1/80.

If the titre was reduced by more than one dilution by BES, but a titre of at least 1/80 was still obtained, and provided a sufficient reduction in titre was obtained with *B. argentina* antigen, the reaction was classed as "positive partially inhibited by BES".

A reaction was classed as "non-specific, due to unknown antigen" if the reduction in titre after inhibition with *B. argentina* antigen was less than three dilutions. Some samples showing this type of reaction also showed agglutination to the same degree in the formalinised cell and tannic acid-treated cell control.

If after inhibition with BES the titre dropped to below 1/80 the reaction was classed as "non-specific due to BES". Samples showing a titre of less than 1/80 (before inhibition) were regarded as negative.

Transmission Tests

Calves 3 weeks to 6 months of age were splenectomised before use, and the time from drawing of blood to inoculation was never greater than 24 hours. The volume of blood used for inoculation was between 250 and 500 ml (2 to 4 ml per pound body weight).

Blood smears were examined daily for 28 days, and transmission tests were classed as positive only if parasites were found in the smears.

Results

Experimental Infections

Three non-splenectomised calves were infected with *B. argentina* by blood inoculation and then tested daily to determine how soon antibody could be detected. Two calves became positive

to the IH and CF tests on the 7th day, the other animal on the 8th day. Titres rapidly rose to over 1/2,500 for the IH test and to 1/640 and over for the CF test.

Three other calves similarly infected with *B. argentina* still showed positive reactions to both tests after 12, 15 and 22 months respectively. The calf infected for 22 months still had a CF titre of 1/80 and IH test titre of 1/2,560. It was still infected at this stage as was shown by transmission test.

IH Tests on CF Positive Serums

Serum samples from 141 animals showing a positive CF reaction (before absorption with BES) were subjected to the IH test. These animals were tested for infectivity at the same time by transmission tests. They were all field cases but were not a representative sample of positive reactors to the CF test. They were selected so as to give as wide a variety of CF reactions as possible, especially of the false-positive type. The results are summarised in Table 1.

Of 58 known infected animals positive to the CF test, 57 were positive to the IH test. The animal that failed to react to the IH test showed a CF test titre of only 1/10.

The IH test titres ranged from 1/80 to over 1/20,000, whereas the CF test titres ranged from 1/10 to 1/1,280.

Of the 69 animals falsely positive to the CF test, 17 were also positive to the IH test. In addition, 2 animals out of 14 that were initially positive to the CF test but negative when absorbed with BES, showed positive reactions to the IH test.

Field Survey Using CF and IH tests

A group of 2,661 animals on 26 holdings in the Tick Quarantine Area of New South Wales, was tested by both tests. All animals showing

TABLE 1

Babeseia argentina — *Results of Indirect Haemagglutination Tests on Animals showing Positive Complement Fixation Reactions prior to Absorption with Bovine Erythrocyte Stroma Antigen (BES)*

Transmission Test	Complement Fixation Test	Number of Animals	Indirect Haemagglutination Test				Number of Animals Negative (Titre before Inhibition less than 1:80)
			Number of Animals Positive		Number of Animals Showing Non-specific Reactions		
			No Inhibition by BES Antigen	Partial Inhibition by BES Antigen	Complete Inhibition by BES Antigen	Due to Unknown Antigen (no Inhibition by BES or *B. argentina* Antigen)	
Positive	Positive	57	56	0	0	0	1
	Positive (partial absorption by BES antigen)	1	0	1	0	0	0
Negative	Positive	45	10	1	2	7	25
	Positive (partial absorption by BES antigen)	24	3	3	4	0	14
	Initially positive but completely absorbed by BES antigen	14	0	2	3	0	9

positive reactions to either test before absorption or inhibition, were checked by transmission.

The results are shown in Table 2.

The IH and CF tests detected 12 animals that were confirmed as being infected. In addition, one infected animal was positive to the IH test only, giving a titre of 1/80. These positive animals were from six holdings.

Of 11 animals (0.41%) falsely positive to the CF test only one was positive to the IH test. Seven other animals (0.26%) were falsely positive to the IH test but negative to the CF test.

Thirty-three CF negative animals (1.24%) showed nonspecific reactions to the IH test, as did another 4 (0.15%) showing an initial reaction to the CF test.

In an attempt to check the efficiency of these tests for detecting infected animals, the holding with the highest incidence of confirmed infected animals was selected. Out of 52 animals on the property, four were infective on transmission test. The remaining 48 animals were bled, 20 ml samples being taken for injection into a splenectimised calf after pooling. The calf receiving the blood showed no evidence of infection.

Cross Reaction with Babesia bigemina

Two non-splenectomised calves infected with *B. bigemina* by blood inoculation showed positive *B. argentina* IH tests for 3 and 6 weeks respectively. The maximum titres involved were low, one being 1/80 and the other 1/160.

TABLE 2

Results of a Field Survey of 2,661 *Animals on* 26 *Holdings using the Indirect Haemagglutination and Complement Fixation Tests for* Babesia argentina

Transmission Test	Complement Fixation Test	Number of Animals	Indirect Haemagglutination Test				Number of animals Negative (Titre before Inhibition less than 1:80)
			Number of Animals Positive		Number of Animals Showing Non-specific Reactions		
			No Inhibition by BES Antigen	Partial Inhibition by BES Antigen	Complete Inhibition by BES Antigen	Due to Unknown Antigen (no Inhibition by BES or *B. argentina* Antigen)	
Positive	Positive	12	12	0	0	0	0
	Negative	1	1	0	0	0	0
Negative	Positive	6	0	0	0	0	6
	Positive (partial absorption by BES antigen)	5	0	1	0	0	4
	Initially Positive but completely absorbed by BES antigen	13	0	1	4	0	8
	Negative	39	3	3	23	10	0
	Anticomplementary	4	0	0	0	0	4
Not tested*	Negative	2581	0	0	0	0	2581

*A group of 48 of these animals was tested by transmission using 20 ml samples pooled before injection into a splenectomised calf. The test was negative.

Cross reactions with *B. bigemina* infection are not a problem in New South Wales, as both diseases are dealt with by the same quarantine restrictions.

Discussion

The IH test for *B. argentina* has a sensitivity similar to that of the CF test. Of 71 known infected animals listed in Tables 1 and 2, 70 were positive to each test.

It was difficult to judge the efficiency of the IH test for detecting infected animals showing a suspicious (titre 1/5) or negative reaction to the CF test. Because of the low level of infection in the population, it was difficult to obtain such a group of animals. However, of the 70 CF-positive animals tested, 17 had a titre of only 1/10.

Both tests seemed to have similar specificity when compared on the basis of false-positive reactions, that is transmission test negative; no inhibition or absorption or partial inhibition or absorption with BES. The CF test showed 0.41% of this type of reaction in the field survey, while the IH test showed 0.30%.

More non-specific reactions were encountered with the IH test (1.39%) than with the CF test (0.49%). This group comprises those animals negative to transmission tests, and where complete absorption or inhibition with BES took place. It also includes those where the reaction was thought to be due to an unknown antigen in the IH test. These non-specific reactions are not a great problem, as we have found no evidence of such animals being infected. With the IH test the majority of these reactions are of a low titre.

It is difficult to explain the non-specific reactions due to unknown antigen in the IH test. Chordi *et al* (1964) encountered a similar reaction with the toxoplasmosis haemagglutination test. Of the 17 animals (in Tables 1 and 2) showing this type of reaction, five showed reactions equally as strong in the formalinised and tannic acid-treated cell controls. Two other animals showed equivalent reactions in the tannic acid-treated cell control only. The remaining ten animals (all with titres of 1/80) showed no reaction in either control.

The IH test was of some value in eliminating false-positive reactions to the CF test. Altogether, 80 such animals were tested, and only 18 were positive to the IH test. The remainder were either negative or non-specific. Only one animal out of 2,661 in the field survey group was falsely positive to both tests, indicating the low incidence of this type of reaction.

Summary

An indirect haemagglutination (IH) test for diagnosing *Babesia argentina* infection in cattle was described. Formalinised sheep erythrocytes treated with tannic acid were sensitised with an extract of the parasite-erythrocyte stroma mixture used as antigen in the complement fixation (CF) test for *B. argentina*. This antigen was also used to inhibit the reaction in a haemagglutination inhibition test.

Bovine erythrocyte stroma antigen (BES) prepared from uninfected blood was also used to inhibit the IH test and to absorb serums before CF testing. By doing this it was possible to remove some of the non-specific reactions.

The sensitivity and specificity of the IH and CF tests were found to be similar, 70 out of 71 known infected animals being positive to each test. After absorption or inhibition with BES, 0.41% of 2,661 animals were still falsely positive to the CF test and 0.30% to the IH test. Reactors were checked by transmission tests.

False-positive reactions to the CF test are a problem in New South Wales, where they occur up to ten times more often than true positive reactions. Of 80 animals falsely positive to the CF test, only 18 were positive to the IH test, indicating the value of this test in eliminating this type of reaction. The overall incidence of animals falsely positive to both tests was not high, as only one out of 2,661 animals was encountered in the field survey described.

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References

Boyden, S. V. (1951)—*J. exp. Med.* **93:** 107.
Butler, W. T. (1963)—*J. Immun.* **90:** 663.
Chordi, A., Walls, K. W. and Kagan, I. G. (1964)—*J. Immun.* **93:** 1024.
Daniel, T. M., Weyand, J. G. Jr. and Stavitsky, A. B. (1963)—*J. Immun.* **90:** 741.
Jacobs, L. and Lunde, M. N. (1957)—*J. Parasit.* **43:** 308.
Lewis, W. P. and Kessel, J. F. (1961)—*Arch. Opthal.* **66:** 49.
Mahoney, D. F. (1962)—*Aust. vet. J.* **38:** 48.
Mahoney, D. F. (1964)—*Aust. vet. J.* **40:** 369.
Stavitsky, A. B. (1954) — *J Immun,* **72:** 360.
Weinbach, R. (1958)—*Schweiz. Z. allg. Path. Bakt.* **21:** 1043.

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