# THE USE OF A SLIDE AGGLUTINATION TEST TO DEMONSTRATE ANTIGENIC DIFFERENCES BETWEEN *BABESIA BIGEMINA* PARASITES

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## Introduction

A parasitised erythrocyte agglutination test for *Babesia argentina* (Curnow 1968) has been used to show that each relapse population of this parasite in the blood of individual calves contains a specific agglutinogen on the surface of infected cells (Curnow 1973). The transmission of each relapse variant from an animal through ticks caused these variable agglutinogens to revert to a common antigenic type. The antigen that appeared after tick transmission seemed to be strain specific.

Using challenge experiments Callow (1964) reported the occurrence of a strain specific immunity occurring after *B. bigemina* infections in calves. In an effort to demonstrate similar differences by serological means, an agglutination test using an antigen prepared from parasites rather than whole parasitised erythrocytes was developed. This paper describes this test and discusses its possible use in the identification of strains and in the diagnosis of infection.

## Materials and Methods

*Definitions*

*Isolate* — A segment of the wild population of organisms isolated by transference into artificial conditions of maintenance (Lumsden 1965).

*Strain* — The population of organisms derived from an isolate and made to reproduce continuously by serial passage in the host at the laboratory (Lumsden and Hardy 1965).

*Calves* — Calves used in these studies were pure or crossbred Jerseys or Guernseys, 6-12 months of age.

*Estimation of Parasitaemia in Non-Splenectomised Calves*

Parasitaemias in non-splenectomised calves were estimated daily by the thick blood film technique of Mahoney and Saal (1961).

*Preservation of Parasites*

Blood was collected from splenectomised calves when at least 1% of the erythrocytes were parasitised. After the addition of dimethyl sulphoxide to a final concentration of 8%, the blood was frozen rapidly to −79°C in 2 ml amounts in sealed glass ampoules. The ampoules were stored under solid carbon dioxide and the parasites were retrieved from preservation by thawing the contents of the ampoules by immersion in a waterbath at 37°C.

*Transmission of Parasites Through Ticks*

The Wollongbar laboratory strain of *Boophilus microplus* was used throughout as many tests had shown that it was naturally free of *Babesia.*

Splenectomised calves were infested with 10,000 larvae of *B. microplus,* and inoculated intravenously with blood containing the particular parasites so that the peak of the initial parasitaemia coincided with the final engorgement of the ticks. Replete female ticks were collected over a three day period when the parasitaemia in the calf was at least 1%. Tick cultures were maintained at 28°C in humidified (95%) incubators. Eggs from the females of each batch were mixed and 20,000 of the resulting larvae were used to infest non-splenectomised calves. Thick blood films were examined daily from these calves to monitor parasitaemias.

*Parasites Used to Prepare Antigens*

Isolates and strains were named after their place of origin, namely Knockrow, Piggabeen, Wyndham Creek and Mullumbimby. Isolates were collected in the field from animals infected subclinically, and injected into splenectomised calves at the laboratory. When a parasitaemia of 1% was reached in these calves, blood was taken and preserved to provide a stock of the isolate.

Non-splenectomised calves were infected with these isolates, and the parasites were passaged to fresh calves every four months. Strains used in these experiments were taken from these calves after two years.

Tick transmitted (TT) parasites were collected on the third day of the initial parasitaemia in tick infested calves.

*Antiserums*

Antiserums to TT parasites were collected from tick-infested calves one week after the peak of the initial parasitaemia had been reached and before the first relapse. Antiserums were stored at −15°C without the addition of preservative.

*The Slide Agglutination (SA) Test*

*Preparation of antigens* — Antigens for the SA test were prepared from the bloods of splenectomised calves which had been infected with the particular *B. bigemina* parasites. Blood for antigen was collected into EDTA* when the parasitaemia reached 5%. It was centrifuged, the plasma discarded, and the cells washed three times in isotonic sodium chloride solution. The packed erythrocytes were lysed with seven volumes of 0.35% sodium chloride solution and the mixture was then centrifuged at 10,000g for 15 minutes. The upper, darker layer of sediment was retained as the parasite

*EDTA — Ethylene diamine tetra acetic acid, disodium salt used at 1.2 grams per litre of blood.

TABLE 1

*Slide Agglutination Test Reactions Obtained in Cross Tests between Antigens and Antiserums of two Tick Transmitted Isolates of* Babesia bigemina

| Antigen | No of Reactions of Grade | | | | |
|---|---|---|---|---|---|
| | ++++ | +++ | ++ | + | − |
| Homologous | 10 | 2 | 0 | 0 | 0 |
| Heterologous | 1 | 2 | 5 | 1 | 3 |

concentrate. Giemsa† stained smears of this fraction showed intact parasites entangled in erythrocyte stromata.

To prepare the antigen, Giemsa stain was diluted 1/10 in buffered saline prepared by adding one part of pH 7.4 0.01M phosphate buffer to nine parts of 0.35% sodium chloride solution. This stain was then added to an equal volume of the parasite concentrate, and staining was allowed to proceed for 10 minutes. The parasites were then recovered by centrifugation and resuspended in an equal volume of 0.85% sodium chloride solution. Formalin was added to a final concentration of 0.2% and the antigen was stored at 5°C.

*Testing technique* — The actual test was carried out on sheets of glass or microscope slides. One drop of antigen was mixed with an equal volume of undiluted serum. The reagents were mixed by a rotary motion for two minutes. Reactions were graded as follows:—

++++ — Large clumps easily visible with the naked eye appearing within one minute.

+++ — Smaller clumps visible with the naked eye but taking up to two minutes to appear.

++ — A fine granular appearance with small clumps visible when viewed by a 10x hand lens.

\+ — Small clumps when viewed by a 10x hand lens.

− — Complete absence of clumps under 10x magnification.

*The Complement Fixation (CF) Test*

The CF test was carried out by the method described by Mahoney (1962). A titre of 5 was considered to be significant.

**Experimental Procedures and Results**

The Knockrow and Piggabeen isolates were transmitted through ticks to non-splenectomised calves (the Knockrow isolate to calves 1 to 9 and the Piggabeen isolate to calves 10, 11 and 12). On the third day of the initial parasitaemia in calves 9 and 12, blood was collected and inoculated into splenectomised calves and SA antigens A and B were prepared from them.

Antiserums to the TT parasites were collected from calves 1-12 and were tested with antigens A and B by the SA method. The results are shown in Table 1. In tests between antigens and antiserums of the same TT isolate (homologous tests) all reactions were either ++++ or +++ but in heterologous tests only three reached these grades, six were either ++ or + and three were negative.

Other antigens, designated C, D and E, were prepared from the Knockrow, Wyndham Creek and Mullumbimby strains respectively by taking blood from animals subclinically infected with these strains and injecting it into splenectomised calves. Serums were collected monthly from calves 1-5 for a period of 9 months after infection and were tested with antigens A, B, C, D and E. These serums were also subjected to the CF test using antigens prepared from the Knockrow TT parasites. The results of these tests are shown in Table 2.

All serums collected from the five calves reacted with homologous antigen A. Thirty-five had reactions graded as ++++ or +++, the remainder being either ++ or +. All but three of the serums were positive with antigen B (Piggabeen TT isolate) but only 18 were graded as ++++ or +++. With antigen C, taken from the strain derived from the Knockrow isolate, only 31 samples were positive and 7 were graded as +++. All serums were negative with antigens D and E. Twenty of the 45 samples were positive to the CF test.

Serum samples collected during field surveys in north-eastern New South Wales and south-eastern Queensland between 1964 and 1970 were grouped as follows —

1. Positive CF test samples collected in New South Wales from field animals known to be infected with *B. bigemina.*

2. Serums from cattle in seven herds in an enzootic area of south-eastern Queensland. Almost all of these animals would have been infected with *B. bigemina* at some stage and many would have been regularly reinfected because of the permanent tick populations present on some of the herds.

3. Serums from field animals in New South Wales infected subclinically with *B. argentina.*

4. Serums from animals shown by transmission tests to be free of *Babesia.*

The four groups of serums were tested with antigens A, B, C, D and E. The number of positive reactions in each group is shown in Table 3.

The antigens varied in their reactivity but all were less efficient than the CF test with the first group of serums. Antigen B recorded the highest number of reactions with the serums of groups 1 and 2, but it also recorded the highest number of false positive reactions with groups 3 and 4. The strain antigens C, D and E gave fewer reactions with serums from infected animals than the antigens of the TT parasites. With the excep-

†Giemsa — Prepared from Giemsa powder (British Drug Houses, Poole, England) by heating 0.5 grams of the powder with 33 ml of glycerol overnight at 60°C, then cooling and adding 33 ml of methyl alcohol.

TABLE 2

*Results of* B. bigemina *CF and Slide Agglutination Tests on a Series of Serums Collected at Monthly Intervals from Five Calves Infected with the Knockrow Tick Transmitted (TT) Isolate*

| Slide Agglutination Test Antigen | Calf No | Slide Agglutination Test Results† Months after Infection | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 0* | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
| A (TT Knockrow isolate) | 1 | — | 4 | 3 | 3 | 4 | 3 | 3 | 3 | 3 | 2 |
| | 2 | — | 4 | 4 | 4 | 4 | 2 | 3 | 3 | 2 | 3 |
| | 3 | — | 4 | 4 | 1 | 3 | 3 | 2 | 2 | 2 | 2 |
| | 4 | — | 4 | 4 | 3 | 3 | 2 | 3 | 4 | 3 | 3 |
| | 5 | — | 4 | 3 | 1 | 3 | 3 | 3 | 3 | 3 | 3 |
| B (TT Piggabeen isolate) | 1 | — | 2 | 3 | 2 | 2 | 1 | 3 | 3 | 2 | 2 |
| | 2 | — | — | 3 | 3 | 2 | 3 | 2 | 2 | 2 | 2 |
| | 3 | — | — | 4 | 3 | 2 | 2 | 3 | 2 | 3 | 3 |
| | 4 | — | 3 | 3 | 3 | 2 | 3 | 2 | 2 | 2 | 3 |
| | 5 | — | 2 | — | 2 | 2 | 3 | 2 | 2 | 3 | 2 |
| C (Knockrow strain) | 1 | — | — | — | — | — | 4 | 3 | 3 | 3 | 1 |
| | 2 | — | 3 | 3 | — | 2 | 2 | — | 1 | 2 | — |
| | 3 | — | 2 | 3 | 2 | 2 | 1 | 1 | 1 | 1 | — |
| | 4 | — | 2 | 1 | 2 | 2 | — | — | 2 | 2 | 2 |
| | 5 | — | 1 | — | — | — | 1 | 1 | 2 | 2 | — |
| D and E (Wyndham Creek and Mullumbimby strains) | 1 | — | — | — | — | — | — | — | — | — | — |
| | 2 | — | — | — | — | — | — | — | — | — | — |
| | 3 | — | — | — | — | — | — | — | — | — | — |
| | 4 | — | — | — | — | — | — | — | — | — | — |
| | 5 | — | — | — | — | — | — | — | — | — | — |
| Reciprocal CF titres | 1 | — | 160 | 80 | — | — | — | — | — | — | — |
| | 2 | — | 160 | 80 | 20 | — | — | — | — | — | — |
| | 3 | — | 320 | 40 | 40 | — | — | — | — | — | — |
| | 4 | — | 80 | 40 | 10 | — | — | — | — | — | — |
| | 5 | — | 160 | 80 | 10 | 10 | 10 | 10 | 20 | 10 | 5 |

*before infection.
†the symbols 4, 3, 2, 1 refer to ++++, +++, ++ and + reactions respectively.

tion of antigen B, the SA test antigens and the CF antigens were similar in the number of false positive reactions they produced.

### Discussion

The tests carried out between the antigens and antiserums of the TT Knockrow and Piggabeen isolates showed that the SA test detects antigenic differences between *B. bigemina* parasites. All homologous tests had reactions graded as ++++ or +++ while only 3 of 12 heterologous reactions were of comparable strength. However, 9 of 12 heterologous tests showed agglutination suggesting similarity or sharing of

TABLE 3

*The Results of Testing Four Groups of Field Serums with Five Antigens by the* B. bigemina *Slide Agglutination (SA) Test*

| Group of Serums | No of Serums | No Positive to CF Test | No of Serums Positive to SA Test Antigen | | | | | With Any of Antigens Used |
|---|---|---|---|---|---|---|---|---|
| | | | A | B | C | D | E | |
| 1. *B. bigemina* infected group | 117 | 117 | 72 | 81 | 56 | 72 | 49 | 106 |
| 2. From an enzootic area of Queensland | 352 | 249 | 253 | 305 | 164 | 103 | 136 | 333 |
| 3. *B. argentina* infected group | 40 | 3 | 1 | 3 | 0 | 1 | 0 | 4 |
| 4. Uninfected group | 350 | 3 | 2 | 11 | 3 | 2 | 3 | 15 |

some antigenic determinants between the TT isolates. Because of the possibility of species specificity, the ability of the test to detect subclinical infections was investigated.

The strength of the strain specific component of the test was demonstrated by comparison of the results obtained with tests of the antiserums from calves 1-5 using homologous and heterologus TT antigens and strain antigens. With homologous antigen A, strong reactions graded either ++++ or +++ were obtained throughout most of the nine months that the serums were collected. With heterologous TT antigen B the reactions were generally weaker and antigen C prepared from the strain derived from the Knockrow isolate gave weaker reactions still. Possibly antigenic changes occurred during the two years the strain was maintained and allowed to reproduce in the series of calves. Antigenic changes have been shown to occur in chronic *B. argentina* infections (Curnow 1973). The failure of antigens D and E to elicit any reactions suggests that if a species specific component is present it may be weak.

As a diagnostic test all five antigens were less effective with the first group of serums than the CF test was (Table 3). The variability of antigen reactivity with this group was marked. With groups 1 and 2 more positive reactions were obtained with the TT antigens than the strain antigens. This suggests that a wider variety of antigens or a greater amount of antigenic material might be present on the parasites after tick transmission.

The incidence of reactions with any of the 5 antigens used against group 1 serums was higher (106/117) than with any single antigen indicating that the use of a series of antigens might increase the sensitivity of the test. In doing this, there would be a sharp increase in the incidence of false positive reactions as shown by the results with group 4 serums. Using antigens singly and with the exception of antigen B the incidence of false positives was equivalent to that of the CF test and acceptable for the use of the test in New South Wales. This test appeared to be specific for *B. bigemina* antigens as the incidence of reactions with *B. argentina* antiserums was low.

At present the specificity of the SA test is not broad enough to enable it to be used in the general diagnosis of *B. bigemina* infections in New South Wales. With calves 1-5 (Table 2) the SA test using the TT antigens was more sensitive than the CF test. In herds in which infection builds up after a single introduction and antigenic diversity is restricted, the SA test might be useful especially if TT antigens from the same herd were used. The simplicity of the SA test and its use in the field are factors in its favour.


### Summary

A slide agglutination test for *Babesia bigemina* is descirbed. The antigen was a Giemsa-stained and formalinised suspension of parasites prepared by lysing parasitised erythrocytes. The test was carried out by adding a drop of antigen to a drop of undiluted serum on a glass slide and mixing for two minutes by a rotary motion.

Two isolates of *B. bigemina* were tick-transmitted (TT) to 12 calves and antigens were prepared from the TT parasites. Testing of these antigens with antiserums to the TT parasites gave strong reactions in all homologous tests. In heterologous tests 9 of 12 tests showed a reaction but only 3 of these were as strong as in the homologous tests. This demonstrated the test was able to detect antigenic differences between *B. bigemina* parasites.

Using the two TT antigens and three others prepared from strains of *B. bigemina* received from carriers to test a group of subclinically infected animals in New South Wales, the SA test was found to be not as sensitive as the CF test. However, using a TT antigen to test five calves infected with the homologous isolate, the SA test was superior to the CF test. The SA test may therefore have a use in detecting subclinical infections in recently infected herds where a build up of infection has occurred after the introduction of a single infection and where a homologous antigen can be used.



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### References


Callow, L. L. (1964)—*Nature, Lond.* **204:** 1213.
Curnow, J. A. (1968)—*Nature, Lond.* **217:** 267.
Curnow, J. A. (1973)—*Aust. vet. J.*
Lumsden, W. H. R. (1965)—In "Progress in Protozoology". Excerpta Medica Foundation, Amsterdam.
Lumsden, W. H. R. and Hardy, G. J. C. (1965)—*Nature, Lond.* **205:** 1032.
Mahoney, D. F. and Saal, J. R. (1961)—*Aust. vet. J.* **37:** 44.
Mahoney, D. F. (1962)—*Aust. vet. J.* **38:** 48.



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