

## THE DETECTION OF ANTIBODIES TO *COWDRIA RUMINANTIIUM* IN SERUM AND *C. RUMINANTIIUM* ANTIGEN IN *AMBLYOMMA HEBRAEUM* BY AN ENZYME-LINKED IMMUNOSORBENT ASSAY

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### ABSTRACT

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A sensitive and reliable enzyme-linked immunosorbent assay for the detection of antibodies to *Cowdria ruminantium* in serum and *C. ruminantium* antigen in *Amblyomma hebraeum* nymphae is described. For the screening of antibodies, *C. ruminantium* from *A. hebraeum* nymphae, partially purified by wheat-germ lectin affinity chromatography, was used as antigen. To screen nymph populations, sera from either Ball 3 strain-infected sheep or Kumm-strain infected mice were used. By using appropriate controls the assays were rendered specific with respect to *C. ruminantium*.

### INTRODUCTION

The detection of antibodies to *Cowdria ruminantium* in serum is important for studies on immunological aspects of heartwater (Alexander, 1931; Neitz, 1939; Du Plessis, 1970) in epizootiological investigations and in the diagnosis of the disease (Ilemobade, 1976).

Both Du Plessis (1970) and Ilemobade (1976) had negative or inconclusive results using an indirect fluorescent antibody (IFA) test with squashed brain material as antigen for the detection of antibody to *C. ruminantium* in serum. However, Du Plessis (1981) was successful with an IFA test employing peritoneal cells of mice infected with a mouse infective strain (Du Plessis & Kumm, 1971) as antigen. A simple, useful capillary flocculation test using crude brain material as antigen has also been reported (Ilemobade, 1976; Ilemobade & Blotkamp, 1976).

Since the sensitivity and specificity of serological tests depend on the source and the method employed for the preparation of the antigen as well as on the detection method for antibody-antigen interaction, alternative tests were investigated. In this paper an enzyme-linked, immunosorbent assay (ELISA) is described, using as antigen *C. ruminantium* from *Amblyomma hebraeum* nymphae partially purified by wheat-germ lectin affinity chromatography. In addition, the screening of *C. ruminantium* antigen in *A. hebraeum* nymphae by an ELISA was undertaken. The latter study could be of value in the study of the infection rate of tick populations, which is one of the factors governing the epizootiology of heartwater (Uilenberg, 1983). Furthermore, an indication of the infectivity of nymph suspensions used for vaccination (Bezuidenhout, 1981) could be obtained.

### MATERIALS AND METHODS

Analytical quality reagents were used in all the experiments. All the glassware and equipment were sterilized with 70% (v/v) ethanol and the buffers by filtration through 0,25 µm filters<sup>1</sup>.

#### 1. Detection of antibodies to *C. ruminantium* in serum

##### *Preparation of antigen*

*C. ruminantium* antigen was prepared from infected *A. hebraeum* (Spes Bona strain) nymphae using wheat-

germ lectin affinity chromatography (Viljoen, Vermeulen, Oberem, Prozesky, Verschoor, Bezuidenhout, Putterill, Visser & Neitz (1985). Non-infected nymphae were used as controls.

##### *Preparation of sera*

Sera from 41 heartwater-infected and 14 non-infected (susceptible) sheep and 9 heartwater-infected cattle were used for serum screenings. Sheep were infected with 1 dose of the Onderstepoort Ball 3 heartwater blood vaccine (Bezuidenhout, 1981). Except for one sheep which had been inoculated subcutaneously (sc) the dosage was by intravenous (iv) injection. Animals which showed no rise in rectal temperature during the 21-day observation period, were challenged with the vaccine. Reacting sheep were treated by intramuscular injection with oxytetracycline at a dosage rate of 10 mg/kg. All the sheep were then boosted 17 weeks after the initial inoculation with the vaccine. Sera were collected 5 weeks after the final challenge.

Six lambs under 3 weeks of age were inoculated with the heartwater vaccine and sera was obtained 5-6 weeks later.

One-year-old cattle (9) were injected iv with 2 ml infective nymph suspension (Bezuidenhout, 1981), consisting of 1 nymph per dose. After 8 weeks they were challenged iv with 5 ml of heartwater infective sheep blood. Sera were collected 19 weeks after the initial inoculation.

The blood samples were left to clot and after 4 hours the coagulated blood was centrifuged for 10 min at 300 × g at room temperature in a Piccolo bench top centrifuge. The sera were siphoned off, divided into 1 ml aliquots and stored at -75 °C.

#### 2. Detection of *C. ruminantium* antigen in *A. hebraeum* nymphae

##### *Preparation of nymph crude extracts*

In order to screen *A. hebraeum* nymphae for the presence of *C. ruminantium* Ball 3 strain, nymphae were homogenized individually for 10 min in 1 ml of 0,05 M HEPES, 0,154 M NaCl buffer (pH 7,4), at low speed with an Ultra Turrax<sup>1</sup>. All the preparation steps of the crude extracts were performed at 4 °C. The nymphal homogenates were centrifuged for 30 min at 1 000 × g in a Rotor 40 in a Beckman L5-65 ultracentrifuge<sup>2</sup> which was also used in all subsequent centrifugations.

The supernatants were then centrifuged for 30 min at 10 000 × g in a Rotor 40. The resultant supernatants

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were centrifuged at  $30\,000 \times g$  for 30 min. The final sediment was resuspended in 1 ml of the HEPES buffer. Nymphae which were known to be non-infected were used as controls.

#### Preparation of sera containing antibodies to *C. ruminantium*

Sera from Kumm strain *C. ruminantium*-infected mice (Du Plessis & Kumm, 1971) and sera from Ball 3 strain-infected sheep were used to screen *A. hebraeum* nymph populations. Sera were prepared as described above.

### 3. Protein determinations

Protein was determined by the high temperature, biuret-Folin method described by Dorsey, McDonald & Roels (1977). The Folin Ciocalteau<sup>1</sup> reagent was diluted 1:1 with distilled water. The colour development was monitored at 660 nm in a Beckman Model 25 spectrophotometer against a blank, containing 0,1 ml of HEPES buffer.

### 4. Enzyme-linked immunosorbent assay (ELISA)

A modification of the ELISA method described by Notermans, Timmermans & Nagel (1982) as employed by Viljoen *et al.* (1985) was used.

Serum solutions were made up as follows: 1 ml of serum was diluted 1:30 with a 0,05 M Tris, 0,154 M NaCl buffer, pH 7,4. As antigen, 10 µg of protein/ml of sonified-infected or non-infected nymphal fractions or of wheat-germ lectin column Peak 2 was used. A 1000 × dilution of either rabbit-anti sheep IgG peroxidase<sup>2</sup>, rabbit-anti mouse IgG peroxidase<sup>2</sup> or rabbit-anti bovine IgG peroxidase<sup>2</sup> conjugates in 1 % (w/v) bovine serum albumin/phosphate buffered saline was used for detection.

To 10 ml of the freshly made substrate buffer, containing 1 mg/ml of o-phenylenediamine<sup>3</sup> in 0,1 M citrate buffer, pH 4,5, 5 µl 30 % (v/v) H<sub>2</sub>O<sub>2</sub> was added immediately before use. Colour development was monitored with a Titertek Multiscan MC<sup>4</sup> at 690 and 450 nm for 30–45 min.

The infected to non-infected (P/N) ratios reported for sera screening were calculated by dividing the absorbance value obtained from the lectin fraction (Peak 2) from infected nymphae by the value obtained from the corresponding lectin fraction from non-infected nymphae.

In the case of nymph screenings, P/N ratios were calculated by dividing the absorbance values obtained for nymphae which had been tested by the absorbance value for known non-infected nymphae.

## RESULTS

#### Detection of antibodies in sera

From Table 1 it is evident that of the 41 sheep sera screened for antibodies to *C. ruminantium* after 2–3 inoculations with the Ball 3 strain, 100 % showed P/N ratios of 1,8–2, of which 83 % were above 2. The 6 lambs infected once at under 3 weeks of age and tested 5–6 weeks later, showed lower ratios of 1,6–1,8. All Ball 3 negative sheep sera exhibited P/N ratios of less than 1,05. All of the cattle sera screened exhibited P/N ratios higher than 2. No negative cattle sera were tested.

#### Detection of antigen in nymphae

The results obtained from infected nymphae (Table 2), whether tested with sera from Ball 3 strain infected sheep

TABLE 1 ELISA screening of antibodies to *Cowdria ruminantium* in the sera of sheep and cattle

Material source			
Serum screened	Antigen	P/N ratio	% <sup>1</sup>
41 Ball 3 + sheep	Ball 3 WG (P2)	>1,8	100
		>2	83
6 Ball 3 + sheep <sup>2</sup>	Ball 3 WG (P2)	1,6–1,8	100
14 Ball 3 – sheep	Ball 3 WG (P2)	<1,05	100
9 Ball 3 + cattle	Ball 3 WG (P2)	>2	100

WG(P2) = Peak 2 of wheat-germ lectin column

+ = Heartwater infected

– = Heartwater non-infected

<sup>1</sup> = % of animals showing indicated P/N ratios

<sup>2</sup> = Sheep infected only once as lambs

TABLE 2 ELISA screening for *Cowdria ruminantium* antigen in *Amblyomma hebraeum* (Spes Bona strain) nymphae

Material source			
Antigen screened	Serum	P/N ratio	% <sup>1</sup>
50 Ball 3 + nymphae	Ball 3 + sheep	>1,5	78
		>2	60
		<1,1	22
50 Ball 3 – nymphae	Ball 3 + sheep	<1,1	100
30 Ball 3 + nymphae	Ball 3 + sheep	>1,5	80
		>2	66
	or	<1,1	20
	Kumm + mouse	>1,5	77
		>2	66
		<1,1	23
50 Ball 3 + nymphae	Kumm + mouse	>1,5	74
		>2	60
		<1,1	26
50 Ball 3 – nymphae	Kumm + mouse	<1,1	100

+ = heartwater infected

– = heartwater non-infected

<sup>1</sup> = of ticks showing indicated P/N ratios

or sera obtained from Kumm strain-infected mice, were virtually identical, with at least 74 % of the infected nymphae showing ratios above 1,5 (>60 % exhibited values above 2).

## DISCUSSION

From the results it is evident that a clear indication of the absence or presence of antibodies to *C. ruminantium* in the serum of sheep or cattle is given by ELISA employed in this study. Furthermore, since Peak 2 of wheat-germ lectin (Viljoen *et al.*, 1985) from non-infected nymphae was used as control, the test is specific for *C. ruminantium*. The antigen, obtained from *A. hebraeum* nymphae and partially purified by lectin chromatography (Viljoen *et al.*, 1985), proved to be satisfactory for this assay.

The fact that sera for screening were obtained 5 weeks after the final inoculation suggests that the antibody being tested was IgG. Ilemobade (1976), on the other hand, found by the capillary flocculation test that antibodies were detectable for 1–3 weeks only after treatment, spontaneous recovery or challenge-inoculation of infected animals. He deduced that the antibodies involved were IgM, since they are the first antibodies produced in response to an immunogen and have a relatively short half life (Bauer, Mathies & Stavitsky, 1963).

The screening of nymphae for the presence of *C. ruminantium* by the described ELISA showed that virtually identical results were obtained with either sera from Ball 3 strain infected sheep and sera from Kumm strain infected mice. This confirms the notion of Du Plessis (1981) that the 2 strains are immunologically closely

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related. The observation that 22%–26% of the infected nymphae showed absorbance ratios of less than 1,1 could either be due to the inability of the test to detect the organisms in these ticks or to their absence. Since the nymphae were infected in the larval stage and subsequently fed as nymphae on susceptible sheep (Bezuidenhout, 1981), either the larvae were refractory to infection or the organisms were lost during moulting. Screening of larvae for *C. ruminantium* should be conducted to help solve this enigma. Bezuidenhout, Potgieter & Du Plessis (1985) reported infection rates of between 1%–20% using a fluorescent antibody test and mice as experimental animals.

The ELISA reported in this paper should prove to be of value in epizootiological investigations and in the immunodiagnosis of heartwater. The application of the assay in the determination of the earliest stage of detection of antibody in diseased animals, and the determination of the persistence of antibody are to be explored in further investigations. These studies, in conjunction with the determination of immunity to challenge, could in turn substantiate the finding of Du Plessis that immunity to heartwater is mediated cellularly rather than humorally. (Du Plessis, 1970; 1981).

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#### REFERENCES

- ALEXANDER, R. A., 1931. Heartwater: The present state of our knowledge of the disease. *17th Report of the Director of Veterinary Services and Animal Industry*. Union of South Africa, 89–150.
- BAUER, D. C., MATHIES, M. J. & STAVITSKY, A. B., 1963. Sequences of synthesis of  $\gamma$ -1 macroglobulin and  $\gamma$ -2 globulin antibodies during primary and secondary responses to proteins, salmonella antigens, and phage. *Journal of Experimental Medicine*, 117, 889–907.
- BEZUIDENHOUT, J. D., 1981. The development of a new heartwater vaccine using *Amblyomma hebraeum* nymphae infected with *Cowdria ruminantium*. In: WHITEHEAD, G. B. & GIBSON, J. D. (eds). Tick biology and control, 41–45. Tick research Unit, Rhodes University, Grahamstown, Republic of South Africa.
- BEZUIDENHOUT, J. D., POTGIETER, F. T. & DU PLESSIS, J. D., 1985. Recent developments in the control of tick-borne diseases in South Africa. (Journal to be supplied).
- DORSEY, T. E., McDONALD, P. W. & ROELS, O. A., 1977. A heated biuret-folin protein assay which gives equal absorbance with different proteins. *Analytical Biochemistry*, 78, 156–164.
- DU PLESSIS, J. L., 1970. Immunity in heartwater. I. A preliminary note on the role of serum antibodies. *Onderstepoort Journal of Veterinary Research*, 37, 147–150.
- DU PLESSIS, J. L., 1981. The application of the indirect fluorescent antibody test to the serology of heartwater. In: WHITEHEAD, G. B. & GIBSON, J. D. (eds). Tick biology and control, 47–52. Tick research Unit, Rhodes University, Grahamstown, Republic of South Africa.
- DU PLESSIS, J. L. & KUMM, N. A. L., 1971. The passage of *Cowdria ruminantium* in mice. *Journal of the South African Veterinary Medical Association*, 42, 217–221.
- ILEMOBADE, A. A., 1976. Study of heartwater and the causative agent, *Cowdria ruminantium* (Cowdry, 1925), in Nigeria. Ph.D. thesis, Zaria
- ILEMOBADE, A. A. & BLOTKAMP, J., 1976. Preliminary observations on the use of the capillary flocculation test for the diagnosis of heartwater (*Cowdria ruminantium* infection). *Research in Veterinary Science*, 21, 370–372.
- NEITZ, W. O., 1939. The immunity in heartwater. *Onderstepoort Journal of Veterinary Research*, 13, 245–283.
- NOTERMANS, S., TIMMERMANS, P. & NAGEL, J., 1982. Interaction of staphylococcal Protein A in enzyme-linked immunosorbent assays for detecting staphylococcal antigens. *Journal of Immunological Medicine*, 55, 35–41.
- UILENBERG, G., 1983. Heartwater (*Cowdria ruminantium* infection): Current status. *Advances in Veterinary Science and Comparative Medicine*, 27, 427–480.
- VILJOEN, G. J., VERMEULEN, N. M. J., OBEREM, P. T., PROZESKY, L., VERSCHOOR, J. A., BEZUIDENHOUT, J. D., PUTTERILL, J. F., VISSER, L. & NEITZ, A. W. H., 1985. Isolation of *Cowdria ruminantium* by cellular affinity chromatography and detection by an enzyme-linked immunosorbent assay. *Onderstepoort Journal of Veterinary Research*, (in press).