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THE APPLICATION OF THE INDIRECT FLUORESCENT ANTIBODY TEST IN RESEARCH ON HEARTWATER

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ABSTRACT

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The preparation of the antigen, details of the reagents, the titration of the antispecies conjugates and the execution of the indirect fluorescent antibody test are described. The sensitivity and specificity of the test and its applicability to the detection of antibodies to *Cowdria ruminantium* are recorded. The test is both highly specific and sensitive and can be applied to a wide range of studies on heartwater, including epidemiology, determination of the *C. ruminantium* infection rate of *Amblyomma* ticks and the evaluation of immunization against heartwater. The test can also be used to detect antibodies to the heartwater agent in the sera of game.

INTRODUCTION

The serological tests for the detection of antibodies to *Cowdria ruminantium* that have been recorded in the literature, are summarized in Table 1.

Since the first report on the use of the indirect fluorescent antibody (IFA) test to detect antibodies in the serum of sheep and cattle that had recovered from artificial infection with *C. ruminantium* (Du Plessis, 1981), the test has been used not only to study the epidemiology of heartwater (HW) (Du Plessis, 1982b; Du Plessis, Bezuidenhout & Lüdemann, 1984; Du Plessis, 1987), but also to determine the *C. ruminantium* infection rate of ticks (Du Plessis, 1985a). In the Caribbean it has been successfully employed to determine the prevalence of HW on the islands where *Amblyomma variegatum* occurs (Camus & Barré, 1987).

The test has been improved in the course of these investigations and experience has been gained of its application. It has therefore been decided to update the technical details of the test and to record its applicability, its advantages and shortcomings.

REAGENTS, METHODS AND RESULTS

Antigen

The peritoneal macrophages of mice infected with the Kümm strain of *C. ruminantium* (Du Plessis & Kümm, 1971; Du Plessis, 1982a) were used as antigen. Four- to 6-week-old specified-pathogen-free outbred mice of both sexes were used to produce antigen in this laboratory. Several inbred strains of mice (AKR, C57BL/6 and NMRI) were also used at some stage, but the antigen prepared from these mice had no advantage over that prepared from the outbred mice.

The peritoneal cells used as antigen were collected from mice infected with an infective inoculum with an infectivity titre of at least 10^3, prepared as follows. Ten 4–6-week-old mice were injected intraperitoneally with 0,2 mℓ of stock antigen which had been stored in liquid nitrogen. The infectivity of the stock antigen, usually with an infectivity titre of 10^2–10^3, had to be such that all 10 mice showed clinical signs of ruffled hair coat and dyspnoea not later than the 12th day after infection. The mice were killed by severing the cervical spinal cord, and their livers and spleens were homogenized in buffered lactose peptone (BLP)[1] on an approximate 20 % mass volume basis. Appropriate aliquots of the infective inoculum prepared in this manner were stored in liquid nitrogen.

A sample of the infective inoculum was withdrawn and its infectivity to mice determined by preparing 4 tenfold serial dilutions in BLP and inoculating 5 mice per dilution intraperitoneally with 0,2 mℓ per mouse. The mortality score of the mice was recorded and the titre of infectivity calculated according to the method of Reed & Muench (1938). Only if the titre exceeded 10^3 were five- and tenfold dilutions prepared in BLP and 3 groups of mice inoculated with 0,2 mℓ per mouse of undiluted and five- and tenfold diluted suspensions to determine the suitability of the suspension as an inoculum to prepare antigen.

When the mice in a group started showing the above clinical signs, 2 or 3 mice were killed. The skin of the thorax and abdomen was removed and 1,5–2 mℓ of FA buffer[2] introduced into the abdominal cavity with a 2 mℓ syringe fitted with a 2,5 cm, 20-gauge needle. With the needle held *in situ*, the abdominal contents were gently massaged and the maximum quantity of fluid withdrawn. The pooled peritoneal washings of the 3 mice were centrifuged for 5 min at 1 000 r.p.m., the supernatant fluid was discarded and the cells resuspended in 0,25–0,3 mℓ of buffer to obtain a concentrated suspension of cells.

A smear was prepared from a small drop of the cell suspension, fixed in methyl alcohol and stained for 50 min with 5 % Giemsa at a pH of 7,4. The percentage of parasitized cells for each of the 3 groups of mice was determined and the dilution giving a percentage parasitized cells of 2 % or higher was used for the future preparation of antigen slides.

Groups of 10–20 mice were infected with the inoculum at the selected dilution, and the percentage of infected cells determined each time. If 2 % or more of the cells in the Giemsa-stained smear were parasitized, the cells from the rest of the group were harvested, washed and pooled with those of the mice used to prepare the Giemsa-stained smear. The pooled cells were suspended in 8–10 mℓ of buffer, centrifuged and resuspended in a small volume of buffer, such that a droplet of the suspension placed with a platinum wire loop in one of the wells of a commercially prepared 15-well slide formed a sparse monolayer of cells under low power microscopic examination. The higher the percentage of parasitized cells, the more diluted the suspension.

The antigen slides were air-dried, wrapped in tissue and tinfoil and stored in liquid nitrogen, where they retain their reactivity for more than a year. Antigen slides can be stored at room temperature for 10 days, at 4 °C for 21 days, and at −18 °C for 6–9 months.

Immediately before use, the antigen slides stored in liquid nitrogen were exposed to the air for a few seconds until the frosted surface took on a moist appearance, and

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[1] BLP. 181 g Na_2HPO_4; 26,4 g KH_2PO_4; 30 ℓ distilled water; 2 % Difco peptone; 10 % lactose

[2] FA buffer, pH 7,2. 80 g NaCl; 27 g $HNa_2PO_4 . 12H_2O$; 4 g $H_2NaPO_4 . 2H_2O$; distilled H_2O to 1 ℓ; tenfold dilution used as working solution

TABLE 1 Serological tests for the detection of antibodies to *C. ruminantium*

Test	Source of antigen	Specificity	Sensitivity	Shortcomings	Reference
Capillary flocculation	Infected goat and bovine brain	Presumably good	Poor	+ive for only 1–4 weeks after infection	Ilemobade & Blotkamp, 1976
Complement fixation	Kümm strain infected sheep brain	Good	Poor	Anti-complementary and non-specific activity	Du Plessis, 1982a
Indirect fluorescent antibody	Kümm strain infected mouse peritoneal cells	95 % in experimental infections	90 %	Antigen preparation finicky. Cross reactions with *Ehrlichia*	*Vide infra*
Indirect fluorescent antibody	Infected goat neutrophils	Good	Good	Cross reactions with *Ehrlichia*	Holland *et al.*, 1987
Elisa	Wheat-germ purified Ball 3 strain infected *A. hebraeum* nymphae	55–100 %	100 % for 6 weeks after infection. High titres for longer	Antigen preparation difficult. High background	Neitz *et al.*, 1986

TABLE 2 Sensitivity of the IFA test expressed as the percentage of animals serologically positive after artificial infection

Animal species	No. of animals	Age at infection (months)	No. of serologically positive animals[1]	% positive
Cattle	30	1	28 (3)	93,3
Cattle	20	8	20 (3)	100
Cattle	20	13	19 (2)	95
Sheep	14	6–12	13 (1)	92,8
Goats	30	24–36	22 (2)	73,3
Goats	28	6–12	27 (12)	96,4
Total	142		129	
%			90,8	

[1] In parentheses—months post-infection

TABLE 3 Range of antibody titres detectable with the IFA test 4–6 weeks after infection and their persistence in cattle, sheep and mice

Animal species	*C. ruminantium* strain	Range of titres (reciprocals)	Persistence in months (titre)
Cattle	Ball 3	320–5 120	3–7 (1:20)
Sheep	Ball 3	5 120–20 480	39 (1:80)
	Kümm	5 120–20 480	39 (1:80)
Mice	Tick-derived field strains	10–1 000	—
	Kümm	10 240	18 (1:5120)

then were plunged into cold methyl alcohol for 1–3 seconds. To prevent confluence of drops between wells, the slides had to be dry before the serum was placed in the wells.

Conjugates

Commercial antispecies conjugates were used. Upon receipt, the conjugates were diluted 1:10 in FA buffer and stored in suitable aliquots at this dilution. The working dilution of a conjugate was determined by testing serial two-fold dilutions of the conjugate against serial fourfold dilutions of both control positive and negative sera in a block titration test. The conjugate dilution that gave specific fluorescence with the highest positive control serum dilution was used as the working dilution.

Conjugates are normally stored at 4 °C, rather than at −18 °C because one extra freezing and thawing action is eliminated. The deleterious effects of even a limited number of freezing and thawing actions have repeatedly been found to be most detrimental. The reactivity of conjugates remains unchanged after storage at 4 °C for as long as 18 months. It is advisable, though, to ascertain the reactivity of conjugates from time to time, since defective conjugates or those with a declining reactivity have been found to be one of the most common and frustrating causes of erratic results.

Execution of the test

A drop of serum at the appropriate dilution *(vide infra)* was placed in each well and the slide was then incubated for 30 min in a moist chamber at 37 °C. On the one hand, too much serum in a well will result in confluence between adjoining wells, and on the other, wells may become dry if the drops of serum are too small. Drying of the wells must be avoided at all times.

After the first incubation, the slide was plunged into a dish of buffer and gently stirred for 15 min. The slide was then flooded with the appropriate antispecies conjugate at the correct dilution and again incubated for 30 min at 37 °C. After a second washing for 15 min, it was mounted in 50 % glycerine in buffer under a coverslip and examined under a binocular microscope equipped with a HB050 mercury burner and a BP 390–490 exciter filter.

A control positive and negative serum was included in each slide. Only if several brightly fluorescing colonies of *C. ruminantium* were readily distinguishable in the well with the control positive serum and no fluorescence in the case of the negative control, were the rest of the wells examined.

When reading the results, attention was focused solely on HW agent colonies of characteristic size, shape and

structure in the cytoplasm of the peritoneal macrophages. In this way, interference by non-specific fluorescence of extracellular, granular material, resembling *C. ruminantium*, could be eliminated. The colonies might vary in size from very small to almost the size of the nucleus of the host cell but they were well demarcated. A cell might contain more than one colony. The smaller colonies were round and the larger ones were inclinded to be oval in shape. Whereas the periphery of a colony always fluoresced in the case of a positive reaction, its internal structural appearance varied according to the developmental stage of the inclusion (Du Plessis 1975; 1985a). The dense body stage was seen as a fluorescent homogeneous structure, whereas fully-developed colonies appeared as a fluorescent mosaic, consisting of densely-packed, individual organisms in the form of minute ringlets. The fluorescence appeared to be concentrated on the envelopes of the particles. Intermediate stages, with an irregular internal structure of varying fluorescent intensity and a distinct strongly fluorescing periphery, were also seen. The great majority of inclusions were usually fully developed colonies.

With a control negative serum, the larger colonies could often be seen as poorly distinguishable structures with vague outlines. In cells exhibiting non-specific fluorescence of the cytoplasm *(vide infra)*, the colonies were more distinct and even appeared to fluoresce.

With experience, distinction could usually be made readily between a negative and a positive reaction. In certain circumstances, however, the reading of the results might be difficult in a small number of cases. In the first place, non-specific fluorescence of the cytoplasm of parasitized cells, rather frequent in the case of sera from adult cattle, could be a hindrance, particularly at the serum dilution of 1:20 usually employed in epidemiological surveys. Secondly, sera heavily contaminated with bacteria and other impurities might cloud specifically fluorescing colonies, and thirdly, the reading would be more time-consuming if too low a percentage of antigen cells are infected, particularly in the case of dirty, contaminated sera. All these encumbering factors take on greater importance in the case of sera with only low levels of antibody.

Sensitivity of the IFA test

The sensitivity of a serologic test is its ability correctly to detect diseased or infected animals, negative tests on animals that have been infected being designated as false negatives (Martin, 1984). Applied to the study of HW, the sensitivity of the IFA test is its ability to detect animals that have been infected with *C. ruminantium* either artificially or naturally through the tick, irrespective of whether clinical disease is manifested or not.

Cattle exposed to *C. ruminantium*-infected *Amblyomma* ticks do not necessarily show clinical signs of disease and there is no other parameter to determine whether they have become infected with the HW agent. The sensitivity of the test can therefore only be evaluated by subjecting to the test the sera of animals that have been infected intravenously with an inoculum of known infectivity. The serological reactions of 142 HW susceptible cattle, sheep and goats artificially infected in this manner, is reflected in Table 2. It can be seen that 90,8 % of the sera at a dilution of 1:20 gave a positive reaction. Only 73,3 % of one group of 30 Angora goats, though, were positive. This comparatively low percentage may be related to their apparent immunological incompetence (Du Plessis, Jansen & Prozesky, 1983). If the goats are omitted from Table 2, 95,5 % of the animals were serologically positive. The sensitivity of the IFA test is therefore well in excess of 90 %.

The false negative reactions, as an indication of animals which fail to develop detectable amounts of antibody after being infected, are probably attributable not only to immunological incompetence but are also related to the susceptibility of individual animals or an animal species to a particular strain of the HW agent. Thus, the levels of antibody detected in the serum of mice inoculated with *C. ruminantium*-infected ticks collected in the field varied from trace amounts in sera diluted to 1:10, to titres as high as 1:1 000 (Du Plessis, 1985a). High antibody titres are recorded in goats, sheep and susceptible cattle breeds that develop severe reactions, whereas more resistant cattle breeds that develop only mild febrile reactions, or no reactions at all, have low levels of antibody (J. L. du Plessis, unpublished data, 1984).

Specificity of the IFA test

The specificity of a serologic test is its ability to correctly detect non-diseased animals (Martin, 1984) and, in the HW context, animals that give a negative test because they have neither been infected with *C. ruminantium* through the tick nor artificially. Since domestic ruminants can only acquire natural infection through *Amblyomma* ticks and *A. hebraeum* is strictly geographically confined in southern Africa, the serum of a closed herd of cattle bred and reared in a region where this tick does not occur, should be free from antibodies to *C. ruminantium*. The negative reactions at a dilution of 1:20, recorded without exception in 100 sera from cattle in the Orange Free State where *A. hebraeum* does not occur, therefore indicate an exceptionally high specificity (J. L. du Plessis, unpublished data, 1986).

Good specificity of a serologic test also implies the absence of cross-reactions with immunologically related organisms. The absence of cross-reactions between the mouse-derived peritoneal macrophage antigen and antibodies to *Chlamydia psittaci, Rickettsia conorii, Rickettsia prowazeki, Rickettsia typhi* and *Coxiella burnetti* (Du Plessis, 1982a) therefore adds to the specificity of the IFA test. The chances of false positives as positive reactions with sera from animals that have never been infected with the HW agent are therefore remote.

Levels of antibody detectable with the IFA test

Antibody is detected in the serum of both cattle and sheep at 2 weeks after experimental infection (Du Plessis, 1981; 1982a). It can be seen from Table 3 that high levels were recorded in the serum of cattle at 4–6 weeks after infection and even higher levels in sheep and mice. In the case of sheep, high titres were attained, irrespective of whether they were infected with the Ball 3 or the Kümm strain of *C. ruminantium*. Much lower titres were recorded, however, in mice infected with tick-derived field strains (Du Plessis, 1985) than in mice infected with the Kümm strain.

Antibodies were detectable with the IFA test in the serum of sheep and mice for much longer after infection than in the case of cattle (Table 3). This observation was made both in calves immunized before the age of 1 month and in cattle re-infected once or twice after initial infection at 8 months of age.

The effects of re-infection on antibody levels

Some of the animals used in an experiment designed to study levels of conglutinin in cattle infected with the HW agent (Du Plessis, 1985b), also provided valuable additional information. The effect of re-infection and subsequent challenge on antibody levels was studied in these cattle, as well as the persistence of antibody after re-infection and their susceptibility to the challenge inoculation after varying periods of seronegativity.

Twenty 8-month-old, HW susceptible Bonsmara bull calves were inoculated with *C. ruminantium*-infected sheep's blood (Table 4). They were re-infected 124 days

TABLE 4 Effects of re-infection and challenge on antibody levels

Bovine No.	Reaction category[4]			Reciprocal of FA titre				Interval in days between		
	Infection	Re-infection[1]	Challenge	42 days after infection	At re-infection	28 days after re-infection	60 days after challenge	Re-infection & challenge	Re-infection and seronegativity	Seronegativity and challenge
1	III	IV	IV	320	20	20	—[2]	229	142	87 (1620)[3]
2	IV	III	IV	>320	80	320	20	260	180	80 (1620)
3	IV	IV	III	320	20	20	20	94	170	141 (1620)
4	III	III	IV	320	80	20	20	260	113	92 (540)
5	III	IV	IV	320	80	80	—	229	170	141 (180)
6	IV	IV	IV	320	80	20	20	229	142	115 (540)
7	III	IV	IV	320	80	320	20	260	113	92 (1620)
8	III	IV	IV	320	80	20	20	292	142	115 (180)
9	III	IV	III	320	80	320	<20	292	170	141 (180)
10	III	IV	IV	320	20	80	<20	292	170	141 (540)
11	IV	IV	IV	320	20	80	20	323	170	141 (180)
12	IV	IV	IV	320	80	1 280	20	323	180	175 (180)
13	III	IV	IV	320	80	<20	—	210	<30	<30 (4860)
14	IV	IV	IV	80	20	80	20	292	210	200 (180)
15	III	IV	IV	320	80	20	20	260	180	175 (1620)
16	IV	IV	IV	320	80	80	20	260	180	175 (180)
17	III	IV	IV	320	80	320	20	323	>210	200 (540)
18	IV	IV	IV	320	80	320	20	323	180	175 (1620)
19	IV	IV	IV	320	80	80	20	260	113	92 (540)
20	III	IV	IV	320	80	80	20	260	113	92 (4860)

[1] Interval between infection and re-infection 124 days
[2] Serum not available
[3] (1620) = reciprocal of conglutinin titre at challenge
[4] Animals in Categories III and IV are considered to be resistant (Du Plessis & Bezuidenhout, 1979)

TABLE 5 Correlation between the IFA test and resistance to challenge with *C. ruminantium*

Animals species	No. of animals challenged	IFA positive		IFA negative	
		No. resistant	No. susceptible	No. resistant	No. susceptible
Cattle	153	58	0	57	38
Sheep	19	5	10	0	4
Goats	68	9	20	7	32

later and then challenged at intervals between re-infection and challenge that varied from 94–323 days. Their sera were submitted to the IFA test at monthly intervals after re-infection. The day of challenge was chosen such that the period that elapsed between the first negative test at a serum dilution of 1:20 and the challenge inoculation varied from 3–7 months.

Several conclusions can be drawn from the results in Table 4. Firstly, there was no dramatic rise in antibody levels when these animals were re-infected, since a four- to sixteenfold rise in titre was recorded in the serum of only 9 out of the 20 animals and there was even a drop in the titres of 3 of them. Secondly, in spite of being re-infected, these cattle became seronegative within 4–7 months after re-infection. There was also no increase in the antibody levels of these animals 60 days after they had been challenged. The lack of antibody response both when they were re-infected and when they were challenged could probably be explained by the fact that the IFA test detects antibody to antigen resulting from replication of the HW agent. When *C. ruminantium* was inoculated a second and third time, these animals were resistant, there probably was no or minimal replication of the agent and there was little or no response detectable with the IFA test.

A second important observation that can be made is that not a single animal reacted when they were challenged 3–7 months after having become seronegative. The conclusion that cattle retain a specific immunity against HW for as long as 7 months after detectable levels of antibody have disappeared from circulation must, however, be drawn with caution in the case of animals with serum conglutinin titres higher than 1:320 (Du Plessis, 1985b). It can nevertheless be concluded that at least 7 of these animals were specifically immune to heartwater. The absence of antibody in these animals is consistent with earlier observations that the immunity in HW is cell-mediated (Du Plessis, 1982b; Du Plessis *et al.*, 1984). It must finally be noted that only low levels of antibody were again detected in the serum of these cattle 60 days after they had been challenged, and 2 of them were negative.

Correlation between positive IFA test and immunity

Table 5 shows the serological reaction of 153 cattle, 19 sheep and 68 goats in relation to the number of animals that were either resistant or susceptible to challenge with the HW agent after being infected artificially or naturally through the tick. It can be seen that all 58 cattle that were serologically positive on the day when they were challenged were resistant, whereas only 38 out of the 95 that were serologically negative were susceptible. This means that cattle testing positive to the IFA test can consistently be expected to be immune to artificial challenge, but that serologically negative cattle are not necessarily susceptible to challenge.

While the resistance to challenge of some of the serologically negative cattle can be attributed to a specific cell-mediated immunity, as already pointed out, the resistance of a sizable proportion of them can be ascribed to non-specific factors such as conglutinin (Du Plessis, 1985a).

In the evaluation of the results of the IFA test applied to the epidemiology of HW, the conclusion can be drawn that the serologically positive cattle in a herd, where immunization is not practised, have acquired the infection through the tick and are immune to subsequent challenge. In addition, the animals that have acquired a tick-mediated immunity but no longer have demonstrable levels of serum antibody, and those that have no specific immunity but a non-specific resistance, will also be resistant. Only a section of the cattle that are serologically negative are therefore at risk under field conditions.

The immune status of a herd, expressed as the percentage of animals that are serologically positive in the IFA test, therefore represents a conservative proportion of the herd that would resist tick challenge, a percentage well below that which, in actual fact, would probably resist the challenge.

It can be seen from Table 5 that sheep and Angora goats differ from cattle in this respect. Sixty-seven per cent of sheep and 69 % of the Angora goats that were serologically positive were susceptible to challenge. This difference between cattle on one hand and sheep and goats on the other can probably be attributed to their higher susceptibility to HW and to the absence of non-specific resistance factors such as conglutinin (Du Plessis & Bezuidenhout, 1979).

This finding in small ruminants is also consistent with the view that antibodies play only a minor role, if any, in the immunity to HW (Du Plessis, 1982a; Du Plessis *et al.*, 1984). In epidemiological studies on sheep and Angora goats exposed to tick infection, the IFA test can therefore be used to determine the proportion of a flock that had been infected but not necessarily the percentage animals that would resist challenge.

APPLICATION OF THE IFA TEST

Epidemiological studies

A reliable serologic test to determine the proportion of tick-infected animals in a herd is a prerequisite in any epidemiological survey involving a tick-borne disease. The IFA test has already been employed in 2 epidemiological studies on HW. In the first of these (Du Plessis, 1982b) an attempt was made to determine the relationship between the intensity of tick control and the immunity of cattle to HW. No correlation could be determined, partly because of the inadequacy of information regarding the tick control and no knowledge of the *C. ruminantium* infection rate of the vector tick.

In a second study (Du Plessis & Malan 1987), in which the role played by the tick in determining the immune status of the cattle was investigated, tick counts were carried out on cattle on 23 farms where HW occurs endemically and the immune status of each herd determined with the IFA test. In addition, the infection rate of ticks collected on these animals was assayed by means of a method which also depends on the IFA test (Du Plessis, 1985a). In this study, it was found that the immune status of the cattle was determined largely by the tick loads to which they were subjected and that the influence of the *C. ruminantium* infection rate of the ticks was less evident.

TABLE 6 Evaluation of cattle vaccination by means of the IFA test

Group	Animal description	Immune status	No. vaccinated	No. that reacted	No. of sera tested	No. serologically positive	Recommendation
1	Brahman, adult & calves	Originate from HW endemic area	20	6; 3 died	17	12	Revaccination of seronegatives
2	Adult cows	Originate from HW endemic area	60	15	10	10	Exposure to tick challenge
3	Adult bulls	Susceptible	2	0	2	1	Revaccination of seronegative animal
4	Adult Bonsmara bulls	Previously vaccinated on HW endemic veld	12	4	12	5	Revaccination of seronegatives
5	Adult bulls	Originate from HW endemic area	2	0	2	2	Exposure to tick challenge
6	Adult Brown Swiss bull	Susceptible	1	0	1	1	Exposure to tick challenge

TABLE 7 Game sera submitted to the IFA test

Game species	Origin	No. of sera tested	No. positive	Reciprocals of titres		
				Conjugate		
				Anti-bovine	Anti-goat	Anti-sheep
Impala *Aepyceros melampus*	HW endemic region	14	13	—[1]	10–500	—
Scimitar *Oryx*	Immunized in captivity	1	1	100	—	—
Buffalo *Syncerus caffer*	HW endemic region	11	6	20–100	—	—
Mountain reedbuck *Redunca fulvorufula*	HW endemic region	14	8	—	20[2]	—
Black wildebeest *Connochaetes gnou*	Immunized in captivity	1	1	100	1 000	—
Red hartebeest *Alcelaphus buselaphus*	Immunized in captivity	1	1	20	100	—
Klipspringer *Oreotragus oreotragus*	HW endemic region	3	1	—	20[2]	20[2]
Springbok *Antidorcas marsupialis*	HW endemic region	18	12	—	20[2]	20[2]

[1] Not tested
[2] Tested at 1:20 only

A serum dilution of 1:20 is currently used in epidemiological studies on cattle for 2 reasons. Firstly, it is the lowest dilution at which interference by non-specific fluorescence of the antigen-carrying mouse peritoneal macrophages is minimal. Secondly, at this dilution the number of false negatives is limited to an absolute minimum, since out of 160 serum samples of artificially-infected cattle testing positive at a dilution of 1:20, 19 (12 %) were negative at a dilution of 1:80.

Evaluation of immunization

The IFA test played an important role in a study on the immunity of cattle vaccinated as calves (Du Plessis *et al.*, 1984). The resistance to artificial challenge of animals, vaccinated as calves and subsequently exposed to natural tick challenge, was compared with that of animals also vaccinated but exposed to minimal numbers of ticks. The IFA test on one hand served to monitor the infection rate of the tick-exposed group, and, on the other hand, to prove that, in the other group, only one out of 60 cattle became infected through a tick. In this way, it was possible to conclude that, whereas in the absence of natural tick challenge, vaccination only induces a partial immunity against subsequent artificial challenge, in cattle exposed to even a moderate tick challenge the resistance of animals vaccinated as calves was no better than that of control unvaccinated animals.

The test has also been used on a small scale to evaluate the immune status of valuable cattle after vaccination and prior to being exposed to natural tick challenge (Table 6). The veterinarian in charge, or the owner, is usually in doubt as to whether the vaccinated animals can safely be exposed to tick challenge. In group 2 (Table 6) e.g., only 15 out of 60 animals vaccinated showed a febrile reaction. Since 10 out of 10 randomly chosen sera were positive, the immunity of the whole group was considered satisfactory and the animals were consequently turned out into tick-infested veld without ill effects. In Group 4, only 5 out of the 12 bulls vaccinated were serologically positive and revaccination of the 7 serologically-negative bulls was therefore recommended before they were exposed to tick challenge.

Heartwater in game

Since the possible role of game in the epidemiology of HW is becoming increasingly important, some prelimin-

ary findings on the application of the IFA test on game sera are relevant. It can be seen from Table 7 that with this test antibodies to *C. ruminantium* can be detected in the serum of game animals, either exposed to natural tick infection or immunized artificially. It is interesting to note that in the case of the black wildebeest and the red hartebeest, higher titres were recorded with the conjugate prepared against goat gammaglobulins than with that against bovine gammaglobulins.

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