The detection of antibodies cross-reacting with *Cowdria ruminantium* in the sera of domestic ruminants in regions of South Africa where *Amblyomma hebraeum* does not occur

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ABSTRACT

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High levels of seropositivity, in all probability attributable to *Ehrlichia*, were recorded in the serum of domestic ruminants throughout districts in South Africa where *Amblyomma hebraeum*, the vector of the heartwater agent, does not occur. The antibodies, detected with the indirect fluorescent antibody (IFA) and the indirect ELISA tests, cross-reacted with *Cowdria ruminantium,* which was used as antigen in both tests.

A combination of the IFA and ELISA tests, currently employed to detect antibodies to *C. ruminantium*, facilitates the handling of appreciable numbers of sera and ensures maximum reliability.

INTRODUCTION

Since the appearance of earlier reports that experimentally produced antibodies to several species of *Ehrlichia* react positively in the indirect fluorescent antibody (IFA) test in which either neutrophils (Logan, Holland, Mebus & Ristic 1986; Jongejan, Wassink, Thielemans, Perie & Uilenberg 1989) or the peritoneal macrophages of mice (Du Plessis, Camus, Oberem & Malan 1987) infected with *Cowdria ruminantium* are used as antigen, there have been several reports on cross-reactions in the case of field sera of animals from regions of southern Africa free from *Amblyomma hebraeum.* These cross-reactions were recorded not only with the IFA test in which either infected mouse macrophages or endothelial cell cultures were used as antigen, but also with the indirect and the competitive ELISA tests (Du Plessis, Bezuidenhout, Brett, Camus, Jongejan, Mahan & Martinez 1993) and Western blotting (Du Plessis *et al.* 1993; Mahan, Tebele, Mukwedeya, Semu, Nyathi, Wassink, Kelly, Peter & Barbet 1993). Furthermore, high levels of sero-positivity recorded in cattle and sheep during a limited survey in *Amblyomma*-free areas of South Africa, suggested a widespread infection of domestic ruminants by what would seem to be ehrlichial agents (Du Plessis 1993).

The recent increased demand for boergoats serologically negative for heartwater, for export mainly to the USA and Canada, not only enabled the testing of sera from animals in other parts of South Africa

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where *A. hebraeum* does not occur, but also necessitated a combination of the IFA and the indirect ELISA tests to cope with the large numbers of samples. Although the IFA test may be somewhat more sensitive than other tests (Du Plessis *et al.* 1993), it is true that, on the one hand, the preparation of the antigen for this test is fastidious and the reading of the results sometimes laborious (Martinez, Swinkels, Camus & Jongejan 1990) and, on the other, that the ELISA test is much more suitable for large numbers of samples.

MATERIALS AND METHODS

Sera

The sera of cattle and sheep from *Amblyomma*-free regions subjected to the IFA test in an earlier study (Du Plessis 1993), were also subjected to the indirect ELISA test. In addition, the sera of boergoats destined for export, and born and raised in regions of the Western and Eastern Cape Province where *A. hebraeum* does not occur (Howell, Walker & Nevill 1978), were subjected to both tests.

IFA test

The IFA test, in which the peritoneal macrophages of mice infected with the Kümm stock of *C. ruminantium* (Du Plessis 1982) are used as antigen, was carried out as previously described (Du Plessis & Malan 1987) and subsequently employed (Du Plessis 1993; Du Plessis *et al.* 1993). All sera were tested at a dilution of 1:20.

Indirect ELISA test

Detergent-soluble fractions of *Cowdria* elementary bodies prepared from endothelial cell cultures infected with the Welgevonden stock of *C. ruminantium* (Du Plessis 1985) in the manner described by Soldan, Norman, Masaka, Paxton, Edelsten & Sumption (1993), were used as antigen. The test was carried out as described by Soldan *et al.* (1993), with minor modifications. Immunoplates with the antigen were incubated at 25 °C for 1 h on a shaker. All other incubations were likewise carried out at 25 °C for 1 h on a shaker. Test sera were diluted to 1:25. The reaction to the final staining of the horse-radish peroxidase was not stopped and the optical densities (OD) were recorded at 450 nm in a LST EAR 400AT Easy Reader spectrophotometer. OD readings were multiplied by 1 000 to facilitate the selection of the sera to be subjected to the IFA test. Sera with an OD value of ten below or above the negative control-serum reading were recorded as doubtful. All the sera recorded as negative or doubtful were subsequently subjected to the IFA test. With each batch of sera a number of randomly selected sera with OD values, 10–30 in excess of the negative control-serum reading, were also subjected to the IFA test.

RESULTS

The combined results of the ELISA and IFA tests are given in Table 1. In the case of discrepancy between the outcomes of the two tests, the reaction to the IFA test was used to calculate the percentage positives. It can be seen that, with the exception of ten sheep from the Uniondale district in the Western Cape Province that were all negative, the seropositivity of the sheep and goats varied from 6 % in 93 sheep from the Barkley East district of the Eastern Cape Province to 100 % in both sheep and goats from several districts throughout the country. With the exception of the four districts in the North-Western Province where 60–93 % of cattle were positive, the seropositivity in cattle was distinctly lower than that recorded in small stock from the same area.

A comparison of the results recorded with the two tests shows considerable discrepancy (Table 2). Twenty-one per cent of 24 cattle and 18 % of 80 goat sera that were positive to the ELISA test with OD values of 10–30 in excess of the negative control reading, proved to be negative with the IFA test, whereas 70 % of 54 goat sera recorded as doubtful with the ELISA test, reacted positively in the IFA test. In the case of the ELISA negative sera, the discrepancy was even greater. As many as 43 % of 28 cattle sera and 53 % of 62 goat sera proved to be IFA positive.

DISCUSSION

A high percentage of both cattle and small stock throughout regions of South Africa where *A. hebraeum,* the vector of *C. ruminantium,* does not occur, have antibodies against an agent which must be antigenically closely related to the heartwater agent. Since antibodies to other rickettsial agents such as *Anaplasma marginale, Coxiella burnetti, Chlamydia* and *Rickettsia* spp. do not cross-react with *C. ruminantium* in either the IFA (Du Plessis 1982) or the competitive ELISA tests (Jongejan, Thielemans, De Groot, Van Kooten & Van der Zeist 1991), and in view of the mounting evidence that antibodies to *Ehrlichia* spp. in the sera of naturally infected animals in the field react positively to *C. ruminantium* in several serological tests (Du Plessis *et al.* 1993; Mahan *et al.* 1993), it would seem highly probable that infection with *Ehrlichia* is responsible for the high prevalence of seropositivity.

The widespread occurrence of high percentages of domestic ruminants that harbour these antibodies suggests that there are more than just one tick species acting as vectors. Although there are several

districts (George, Uniondale and Oudtshoorn) from which positive sera originate and where *Rhipicephalus evertsi, Rhipicephalus appendiculatus* and *Hyalomma truncatum* occur (Howell *et al.* 1978), there are seven districts (the four in the North-Western Province, and those of Bloemfontein, Jansenville and Graaff-Reinet) where *R. evertsi* and *H. truncatum* are found, but *R. appendiculatus,* is not (Fig. 1). However, the fact that there are positive sera from four districts in the Eastern Transvaal Province where *R. evertsi,* but neither of the other two species occur, and from the Beaufort-West and Postmasburg districts of the Western and Northern Cape Provinces, respectively, where *H. truncatum* is found, but neither of the other two species, suggests that both *R. evertsi* and *H. truncatum* act as vectors. The implication of the latter is supported by the isolation from an adult *H. truncatum* tick of a putative ehrlichial agent that became more pathogenic and elicited a fatal disease indistinguishable from heartwater after three passages in *A. hebraeum* (Du Plessis 1990). Although in a subsequent study (Du Plessis 1993), the passage in *A. hebraeum* of putative ehrlichial agents isolated from *R. appendiculatus* and *R. evertsi* ticks did not result in dramatic changes in pathogenicity, sero-conversion and resistance to challenge with *C. ruminantium* of sheep on which the *A. hebraeum* ticks had been allowed to feed, suggested that these two tick species are also vectors.

TABLE 1 Percentage domestic ruminants in *Amblyomma*-free regions of South Africa serologically positive to *C. ruminantium* according to the combined results of the ELISA and IFA tests

Province	District	Species			No. of sera tested	% pos.
		Cattle	Sheep	Goats		
Eastern Transvaal	Amersfoort	*			15	20
			*		15	100
	Wakkerstroom	*			15	33
			*		15	100
	Belfast		*		14	71
	Ermelo		*		42	79
North Western Province	Klerksdorp	*			15	66
			*		15	93
	Schweizer-Reneke	*	*		15	93
			*		15	100
					15	100
	Lichtenburg	*			15	80
			*		15	100
	Ventersdorp	*			15	60
			*		15	100
Orange Free State	Bloemfontein	*			16	25
				*	15	100
Kwazulu Natal	Utrecht	*			15	27
			*		15	100
Western Cape	George	*			10	60
			*		10	40
	Uniondale	*			10	40
			*		10	0
	Oudtshoorn			*	35	91
	Beaufort West			*	29	45
Eastern Cape	Barkley East		*		93	6
	Jansenville			*	127	82
	Graaff-Reinet			*	85	98
	Somerset East			*	195	95
	Pearston			*	42	100
Northern Cape	Postmasburg		*		44	70

TABLE 2 Discrepancy in results of cattle and goat sera subjected to both the IFA and the ELISA tests

Species	No. of sera	ELISA			IFA		Discrepancy %
		Pos.	Doubtful	Neg.	Pos.	Neg.	
Cattle	54	24			19	5	21
			2		1	1	
				28	12	16	43
Goats	196	80			66	14	18
			54		38	16	
				62	33	29	53

The ehrlichial agents responsible for the widely distributed seropositivity would appear to be only mildly pathogenic or even non-pathogenic, since the flocks and herds from which the sera were collected were reported to be clinically healthy with no history of mild or transient non-specific clinical signs such as inappetence or loss of condition which might be attributable to ehrlichiosis.

A combination of the IFA and indirect ELISA tests for the serological diagnosis of heartwater and infections due to ehrlichial agents appears to be necessary in order to reduce both false positive and negative reactions, which occur when only the ELISA test is used. There is an element of subjectivity attached to the reading of the IFA test results and the situation not infrequently arises when it is difficult to decide whether an intracytoplasmic colony of *Cowdria* fluoresces or not, particularly in the case of trace amounts of antibody and high levels of cellular background fluorescence. This grey area is much wider in the case of the ELISA test. This was evident from the present study in which a considerable discrepancy between the results of the two tests was recorded. Apart from the apparent narrower margin of error in favour of the IFA test, greater reliance is also placed on this test because an earlier comparative study (Du Plessis *et al.* 1993) already suggested a somewhat higher sensitivity in its favour.

At this institute all sera to be routinely tested for heartwater are currently first screened with the ELISA test. All negative and doubtful samples, as well as positives with OD readings up to 20 above

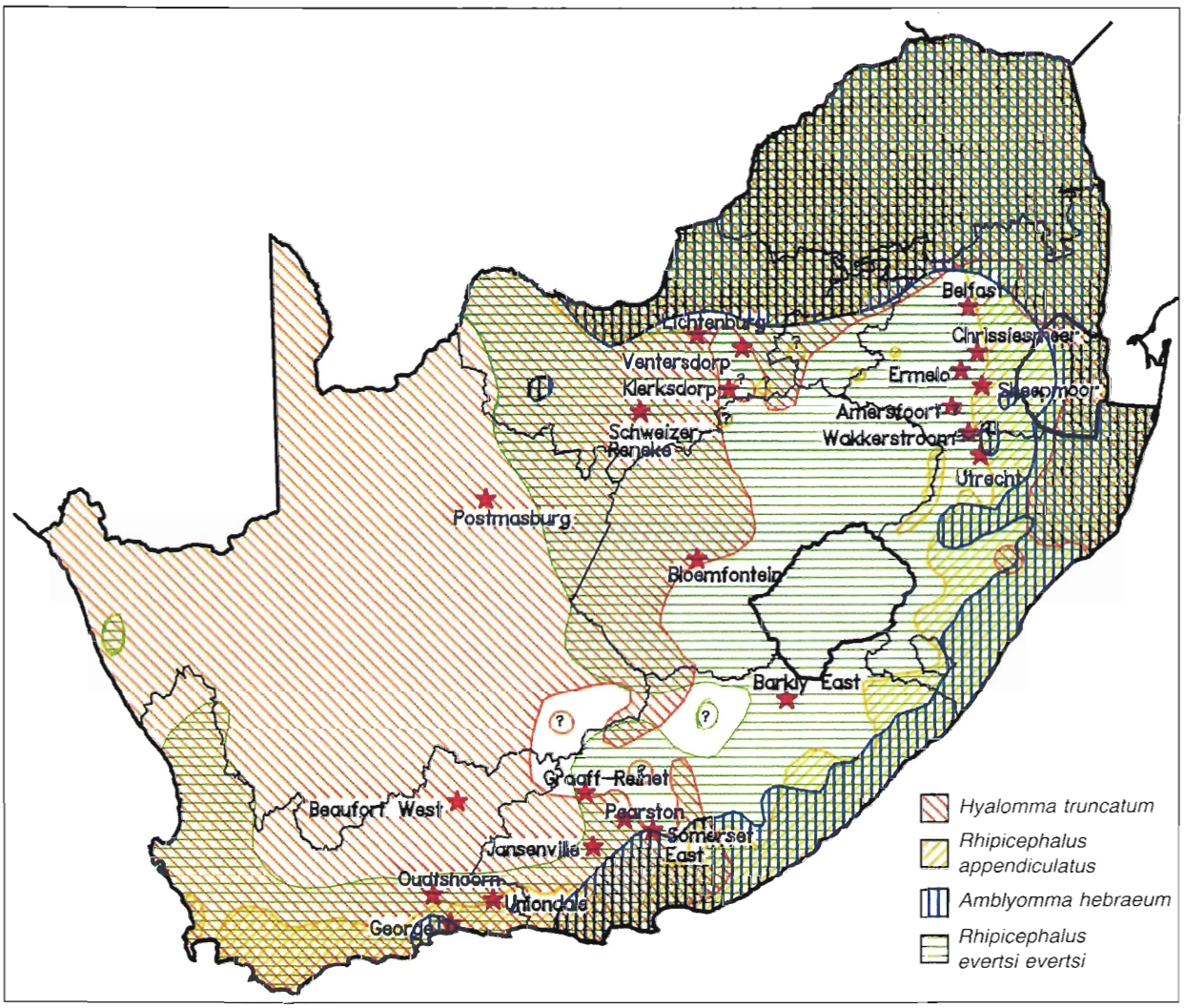

FIG. 1 Districts in *Amblyomma*-free regions where *Rhipicephalus appendiculatis, R. evertsi* and *Hyalomma truncatum* occur, from which serum samples were collected

the negative control value, are subsequently subjected to the IFA test. It is believed that this procedure facilitates the testing of large numbers of sera, eliminates false positive, and minimizes false negative reactions.

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