The use of sucrose-acetone-extracted Rift Valley fever virus antigen derived from cell culture in an indirect enzyme-linked immunosorbent assay and haemagglutination-inhibition test

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ABSTRACT

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A sucrose-acetone-extracted, Madin-Darby-bovine-kidney (MDBK)-derived Rift Valley fever virus (RVFV) antigen was tested both in an indirect ELISA and a haemagglutination-inhibition test for its ability to detect serum antibodies to RVFV. Optimal conditions for antigen concentration, serum and conjugate dilutions for the ELISA were established by checkerboard titration. The specificity and sensitivity of ELISA were determined by the use of paired pre- and post-vaccination sheep-serum samples. Compared with the virus neutralization test, the overall ELISA specificity and sensitivity were 97,4 and 97,3%, respectively. There was a 100% correlation between the results obtained in haemagglutination-inhibition tests with a RVFV sucrose-acetone-extracted antigen derived from hamster liver, and from MDBK cells. A total of 10 582 field-serum samples (84 cattle, 3 659 sheep, 6 839 goats) collected in 1994–1995 from animals of unknown vaccination status in different regions of South Africa were tested with ELISA for antibodies against RVFV. There were no seropositive cattle, 0,16% seropositive sheep and 0,12% seropositive goats. This study demonstrates the potential diagnostic application of cell-culture-derived, sucrose-acetone-extracted RVFV antigen in an indirect ELISA and HI test.

Keywords: Antibody, haemagglutination-inhibition test, indirect ELISA, Rift Valley fever virus, ruminant

INTRODUCTION

Rift Valley fever (RVF) is an acute or peracute, mosquito-borne viral disease of ruminants and humans in Africa, occurring mainly in West Africa and southern Africa. Excessively heavy seasonal rains which favour the breeding of mosquito vectors, particularly after long periods of drought, can cause epidemic outbreaks of the disease in sub-Saharan Africa. The disease is most severe in sheep, cattle and goats, causing high mortalities in neonates and abortion in pregnant animals. Humans become infected from contact with tissues of infected animals or by mosquito bites (Swanepoel & Coetzer 1994).

Although studies have demonstrated the potential for the production of reassortants among multisegmented arboviruses in either vertebrate or vector hosts as well as in cell cultures (Beaty, Sundin, Chandler & Bishop 1985; Battles & Dalrymple 1988; Saluzzo & Smith 1990; Turell, Saluzzo, Tammariello & Smith 1990), there has been no evidence of serological subgroups or major antigenic variation between RVF virus (RVFV) isolates of disparate chronologic and geographic origins (Swanepoel & Coetzer 1994).

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Pathogenic and antigenic cross-reactivity studies in sheep (Swanepoel, Struthers, Erasmus, Shepherd, McGillivray, Shepherd, Hummitzsch, Erasmus & Barnard 1986b) and field studies in cattle (Davies 1975; Swanepoel, Blackburn, Lander, Vickers & Levis 1975; Swanepoel 1976 & 1981) failed to provide any evidence of African phleboviruses other than RVFV which could cause infection or disease in domestic livestock or, alternatively, induce antibodies which could obscure the diagnosis of RVF. Two major epidemics of the disease, the first in 1950 (Alexander 1951), the second in 1974–1976 (Barnard & Botha 1977; Coetzer 1977), as well as several less severe outbreaks and occasional isolations of the virus, have been reported in South Africa (Swanepoel & Coetzer 1994). Since the Egyptian outbreak of RVF in 1977 (Laughlin, Meegan, Strausbaugh, Morens & Watten 1979) and the Senegal-Mauritania outbreak of 1987 (Digoute & Peters 1989; Jouan, Coulibaly, Adam, Philipe, Riou, Le Guenno, Christie, Ould Merzoug, Ksiazek & Digoute 1989), which were both characterized by unusually high morbidity rates and deaths in humans on an unprecedented scale, the spread of RVFV beyond its traditional geographical range and even into the Mediterranean basin (Meegan 1979) remains a very dangerous reality and necessitates accurate and reliable diagnosis of RVF outside Africa and efficient surveillance of the disease in Africa.

Several methods for the detection of antibodies to RVFV have been developed, e.g., the haemagglutination inhibition (HI), complement fixation (CF), indirect immunofluorescence (IF), enzyme-linked immunosorbent assay (ELISA) and neutralization of cytopathic effect in cell cultures (Swanepoel, Struthers, Erasmus, Shepherd, McGillivray, Erasmus & Barnard 1986a). The HI test gave poor results according to some reports (Randal, Gibbs, Aulisio, Binn & Harrison 1962) and it is less sensitive than the plaque-reduction neutralization (PRNT) test (Eddy, Peters, Meadors & Cole 1981). The PRNT, although highly sensitive and specific (Shope, Meegan, Peters, Tesh & Travassos da Rosa 1981), requires cell culture or animal facilities. Furthermore, work with live RVFV outside endemic regions requires special containment laboratories (Scherer, Eddy, Monath, Walton & Richardson 1980). The health hazard posed by the handling of infectious RVFV antigen and the relatively high background readings commonly found with ovine and bovine sera (Meegan, Yedloutschnig, Peleg, Shy, Peters, Walker & Shope 1987) are the main reasons why the ELISA technique has not been used more extensively for routine serodiagnosis of RVF. Poor specificity is the result of high background caused by the use of partially purified antigen and/or incomplete blocking of non-specific adsorption of immunoglobulins to the ELISA plate. Efforts to overcome this problem in sheep serum by a more elaborate purification of RVFV in cell culture, which included centrifugation on sucrose density gradients, proved relatively unsuccessful (Swanepoel *et al.* 1986a). The use of sucrose-acetone-extracted antigen from mouse liver infected with RVFV was also unsatisfactory, because these antigen preparations do not readily adhere to ELISA plates, necessitating an additional step in which anti-RVFV antibodies must first be bound to the ELISA plate to capture the crude antigen (Meegan *et al.* 1987).

In this paper we report on the development of an indirect ELISA and haemagglutination-inhibition test to detect antibodies against RVFV, based on a sucrose-acetone-extracted, MDBK-derived antigen.

MATERIALS AND METHODS

Virus and cells

The 53/74 strain of RVFV, isolated in South Africa during epizootics of the disease in 1974/5 (Barnard & Botha 1977), was serially propagated three times in chicken embryo reticulum (CER)—recharacterized as a hamster line, and ten times in Madin-Darby-bovine-kidney (MDBK) cells with a medium free of bovine serum (BS). The culture medium consisted of Eagles Glasgow Modified medium with 10% BS and antibiotics (penicillin, 100 U/mℓ; streptomycin, 100 µg/mℓ).

Preparation of antigen

Monolayers of MDBK cells prepared in 2 148-cm^2 roller bottles were inoculated with 50 mℓ of BS-free medium containing 0,001–0,005 $TCID_{50}$ of virus per cell. After incubation for 72 h at 37°C and a cytopathic effect (CPE) of 100% of the monolayers, infected cells were suspended in the original culture medium by shaking the bottles, collected and sedimented by centrifugation at 3 000 *g* for 15 min at 4°C. The cell pellet was treated according to the sucrose-acetone extraction method of Clarke and Casals (1958). It was suspended 1:2 (v/v) in a chilled, 8,5% aqueous solution of sucrose and sonicated twice on ice for 30 s at 12 µm in an ultrasonic processor (MSE ultrasonic disintegrator MK 2). The homogenate was dehydrated by means of chilled acetone. The dried sediment was suspended in a volume of saline equal to that of the sonicated suspension, macerated with a syringe, held at room temperature for 1 h and centrifuged at 20 000 *g* for 30 min at 4°C. The supernatant which contained the viral antigen was diluted 1:20 in phosphate-buffered saline (PBS), inactivated with beta-propiolactone (Sever, Castellano, Pelon, Huebner & Wolman 1964), freeze-dried in 0,25-mℓ amounts and stored at 4°C. The safety of the antigen was tested in suckling mice (Shope & Sather 1979). A control ELISA antigen was prepared in a similar manner from non-infected MDBK cells.

The protein concentration of antigens for ELISA was determined by the method of Laemmli (1970).

Indirect ELISA

The procedure, with slight modifications, was based on an indirect ELISA for detecting antibodies to African horsesickness and equine encephalosis viruses (Williams, Du Plessis & Van Wyngaardt 1993). Flat-bottomed microtitre plates (Linbro/Titertek, Flow Laboratories, CT) were coated overnight at 4°C with antigen at a protein concentration of 1–5 µg/mℓ in phosphate-buffered saline (PBS), pH 9,6. After it had been washed, the plate was blocked for 1 h at 37°C with 100 µℓ/well of a blocking solution consisting of 3,5% Nestlé *Lactogen* milk powder (Food & Nutritional Products, Randburg, RSA), 1% casein hydrolysate, and 1% Tween 20 in PBS. This same solution served as diluent for the sera and the conjugate. After it had been washed, 50 µℓ/well of a 1:100 dilution of each serum sample in duplicate wells was incubated at 37°C for 2 h. After it had been washed, 50 µℓ/well of a 1:5 000 dilution of horseradish peroxidase-conjugated protein G (Zymed Laboratories, San Francisco, CA) was added and incubated at 37 °C for 1 h. Following a further wash, 50 µℓ/well of o-phenylene-diamine in citrate buffer (pH 4,0) was added to all wells and the reaction stopped after 20 min by the addition of 50 µℓ/well of 2,0 M H_2SO_4. Optical-density (OD) values were recorded with a microplate reader (Bio-Tek EL340, Bio-Tek Instruments, Winooski, VT, USA) at a wavelength of 492 nm.

ELISA titres were calculated according to the reference-serum (RS) method (Williams 1987) in which a positive reference serum with a known end-titre is used to determine the end-titres of samples according to the following formula:

$$\text{Titre} = \frac{\text{OD test sample} - \text{OD negative control x RS titre}}{\text{OD positive RS} - \text{OD negative control}}$$

The important advantage of this method is that it increases reproducibility of the assay by minimizing test-to-test variations in OD values.

Virus-neutralization test

The microtitre neutralization test (VN) was basically the same as that of Swanepoel *et al.* (1986), except for the 53/74 strain of RVFV and MDBK cells. The titre was expressed as the reciprocal of the serum dilution that completely or almost completely inhibited viral CPE. A serum was considered seropositive when it had a VN titre of ≥1:4.

Haemagglutination-inhibition test

The haemagglutination-inhibition test (HI) was carried out by the use of a micro-technique (Swanepoel *et al.* 1986a). Non-specific inhibitors of haemagglutination were removed by kaolin extraction and adsorption of sera with goose erythrocytes (Clarke and Casals 1958). Non-inactivated sucrose-acetone extracts of RFV-infected hamster liver and RVF-infected MDBK cells were used as antigen. Doubling dilutions of sera were tested against equal volumes of antigen containing 4 HA units. An HI titre of ≥1:10 was considered positive.

Sera

Control sera

Two RVF-susceptible sheep were injected subcutaneously twice, at three-weekly intervals, with 1 mℓ of a freshly prepared 10% suspension of RVFV 53/74 infected suckling mouse brain (Shope & Sather 1979) containing $10^{8,5}$ $TCID_{50}$ of virus. Sheep were bled before vaccination (negative control), 3 weeks after the first vaccination (low positive control) and 3 weeks after the second vaccination (high positive control). The blood was allowed to clot, and the serum was removed and stored in a freeze-dried state. It was reconstituted with distilled water.

Sera from vaccinated sheep

A group of 38 sheep were all vaccinated with a commercially available, inactivated RVFV vaccine (Onderstepoort Biological Products). Seven of these sheep were subsequently vaccinated for a second time. The samples collected from these sheep thus comprised 38 pre- and 38 post-vaccination sera, and seven sera from sheep vaccinated for a second time.

Field sera

A total of 84 cattle, 3 659 sheep, and 6 839 goat sera, collected during 1994–1995 in different regions of South Africa, were tested for antibodies to RVFV in the indirect ELISA. The vaccination status of these animals was unknown.

RESULTS

Standardization of ELISA

Two ELISAs, in which positive and control antigen were utilized respectively, were used to test a known high-positive, a low-positive, and a negative control serum. The OD values of both the positive and low-positive sera varied in a dose-dependent manner when tested with positive antigen over a concentration range of 400–12,5 ng/well, while the negative serum showed minimal reaction (Fig. 1a). All three sera failed to produce any significant signal when tested with the control antigen (Fig. 1b). The optimal antigen concentration was established at 1–5 µg of total protein/mℓ. An ELISA titre of 1:4 000 was taken as the cut-off value between positive and negative

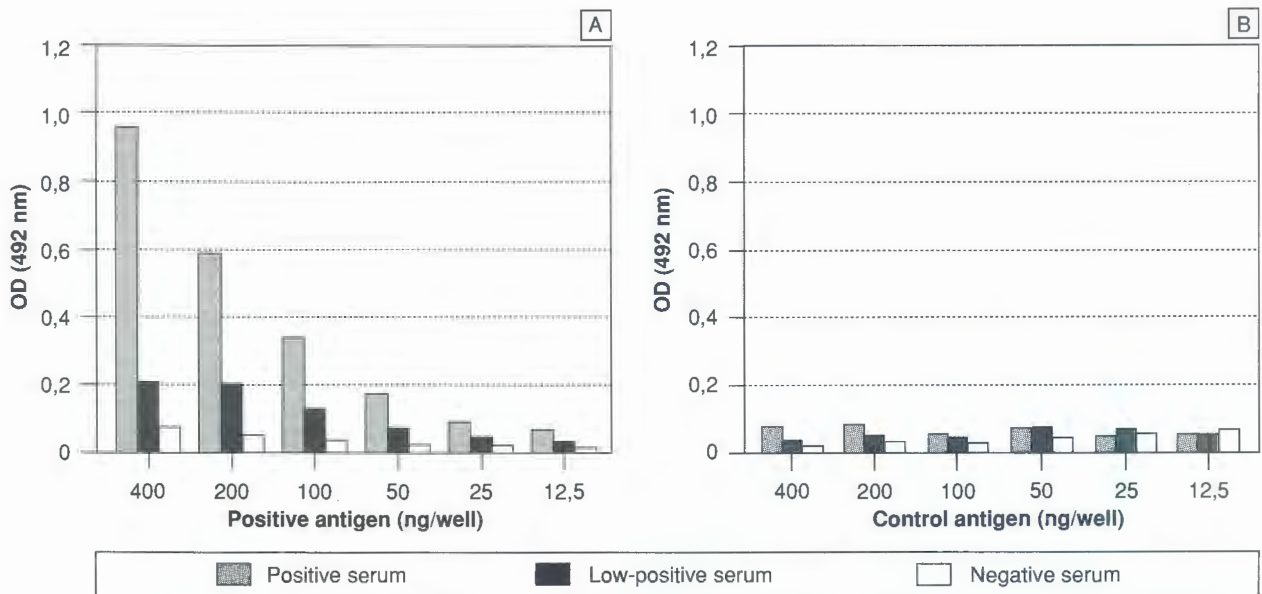

FIG. 1 Standardization of the indirect RVFV ELISA using positive antigen (A) and control antigen (B) with positive, low-positive and negative sheep sera

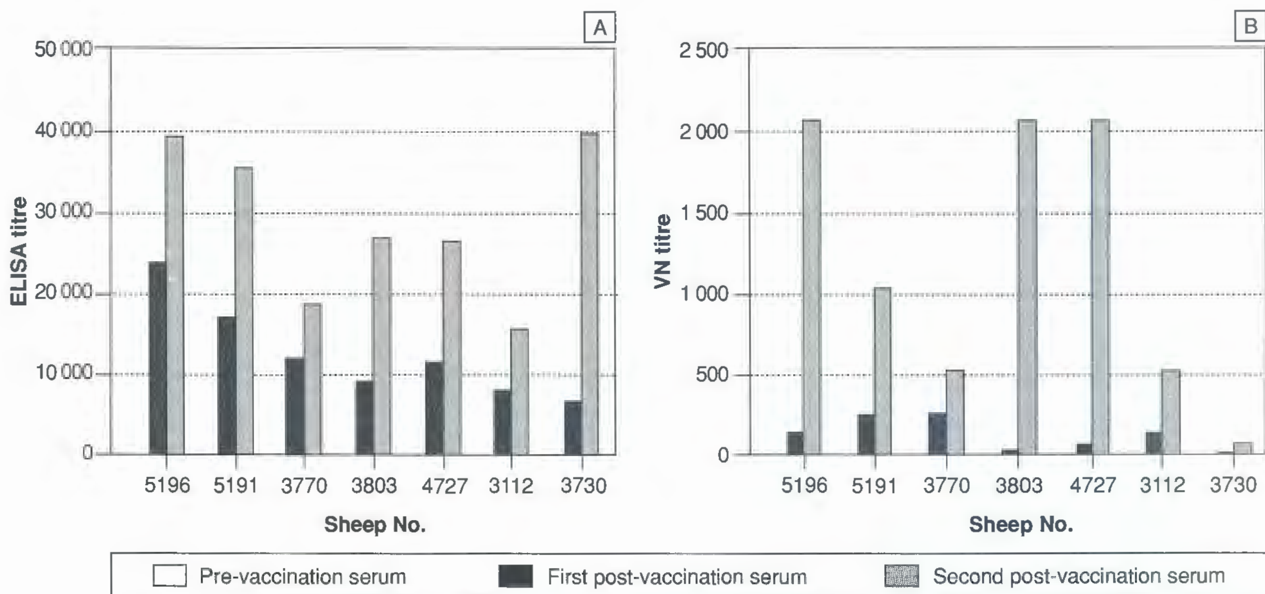

FIG. 2 The immune response of seven sheep to vaccination with an inactivated RVF vaccine, as assayed by the indirect ELISA (A) and the virus neutralization (VN) test (B)

samples, based on the standard deviation of confirmed VN-negative samples.

Comparison of tests

Paired pre- and post-vaccination sera were tested by ELISA, VN and HI tests. The relationship between ELISA, VN and HI antibody titres in pre- and post-vaccination sera after a single inoculation is shown in Table 1. The ELISA (Fig. 2a) and VN (Fig. 2b) titres of seven sheep are shown to demonstrate their immunological response after the first and second vaccinations.

Of a total of 39 VN-negative sera, 38 (97,4 %) tested ELISA negative, and of a total of 37 VN-positive sera, 36 (97,3 %) were ELISA positive (Table 2). One post-vaccination serum (No. 4173), which tested negative

TABLE 1 Comparison of an indirect RVFV ELISA with virus-neutralization (VN) and haemagglutination-inhibition (HI) tests for the detection of antibodies in paired pre- and post-vaccination sheep sera

Sheep No.	Pre-vaccination sera				Post-vaccination sera*			
	HI[a]	HI[b]	VN[c]	ELISA[d]	HI[a]	HI[b]	VN[c]	ELISA[d]
3001	< 10	< 10	< 4	< 3 500	640	80	128	7 300
3013	< 10	< 10	< 4	< 3 500	1 280	160	256	8 500
3112	< 10	< 10	< 4	< 3 500	160	80	128	7 900
3224	< 10	< 10	< 4	< 3 500	160	80	8	6 700
3228	< 10	< 10	< 4	< 3 500	160	80	128	9 200
3429	< 10	< 10	< 4	< 3 500	320	80	16	4 500
3704	< 10	< 10	4	4 000	10	20	8	4 900
3730	< 10	< 10	< 4	< 3 500	40	20	8	6 600
3731	< 10	< 10	< 4	< 3 500	80	20	8	7 300
3747	< 10	< 10	< 4	< 3 500	< 10	< 10	< 4	< 3 500
3770	< 10	< 10	< 4	< 3 500	320	160	256	11 700
3803	< 10	< 10	< 4	< 3 500	40	40	16	9 100
4111	< 10	< 10	< 4	3 700	320	80	16	10 300
4140	< 10	< 10	< 4	< 3 500	< 10	< 10	< 4	< 3 500
4173	< 10	< 10	< 4	< 3 500	< 10	< 10	< 4	6 200
4184	< 10	< 10	< 4	< 3 500	40	20	32	5 300
4187	< 10	< 10	< 4	< 3 500	160	40	8	5 300
4188	< 10	< 10	< 4	< 3 500	10	20	8	4 100
4201	< 10	< 10	< 4	< 3 500	20	20	4	4 500
4210	< 10	< 10	< 4	< 3 500	10	20	16	8 200
4291	< 10	< 10	< 4	< 3 500	1 280	640	256	14 200
4292	< 10	< 10	< 4	< 3 500	80	80	64	6 100
4293	< 10	< 10	< 4	< 3 500	160	320	128	7 700
4341	< 10	< 10	< 4	< 3 500	10	20	4	3 700
4723	< 10	< 10	< 4	3 900	10	20	8	7 900
4727	< 10	< 10	< 4	< 3 500	640	160	64	11 400
4728	< 10	< 10	4	4 700	< 10	< 10	4	5 400
4846	< 10	< 10	< 4	< 3 500	5 120	2 560	1 024	11 600
5032	< 10	< 10	< 4	< 3 500	640	160	128	8 500
5047	< 10	< 10	< 4	< 3 500	320	160	128	10 200
5191	< 10	< 10	< 4	< 3 500	320	160	256	17 000
5196	< 10	< 10	< 4	< 3 500	80	80	128	23 600
6774	< 10	< 10	< 4	< 3 500	40	20	16	7 000
6871	< 10	< 10	< 4	< 3 500	20	20	16	5 000
6902	< 10	< 10	< 4	< 3 500	160	40	32	6 700
6827	< 10	< 10	< 4	< 3 500	5 120	640	1 024	17 100
6940	< 10	< 10	< 4	3 700	320	80	128	7 200
6967	< 10	< 10	< 4	< 3 500	160	80	32	5 300

a = Hamster-liver-derived RVFV antigen used, ≥1:10 titre taken as positive
b = MDBK-derived RVFV antigen used, ≥1:10 titre taken as positive
c = ≥1:4 titre taken as positive
d = ≥4 000 titre taken as positive
* = Sera collected after single vaccination with an inactivated RVFV vaccine

TABLE 2 Analysis of results by indirect ELISA, virus neutralization (VN) and haemagglutination inhibition (HI) tests on 38 paired pre- and post-vaccination sheep sera

Serological assay		No. of sera	
		Pre-vaccination	Post-vaccination
ELISA+	VN+	2	34
ELISA+	VN–	0	1
ELISA–	VN+	0	1
ELISA–	VN–	36	2
ELISA+	HI+	0	33
ELISA+	HI–	2	2
ELISA–	HI+	0	1
ELISA–	HI–	36	2
Total		38	38

in VN and HI tests, was ELISA positive (Table 1). Of 38 sera which tested HI negative, 34 (90,5 %) were ELISA negative, and of 34 HI-positive sera, 33 (97,6 %) were ELISA positive (Table 2). Two pre-vaccination sera (no. 3704, 4728) and two post-vaccination sera (No. 4173, 4728), which tested HI negative, were ELISA positive (Table 2). In only one case did the ELISA fail to detect a very low VN- and HI-seropositive serum (no. 4341).

The titres of the HI test in which antigen extracted from hamster liver was used, were generally higher than those yielded by the HI test in which antigen extracted from MDBK cells was used. When evaluated on a positive/negative basis, however, there was a 100% correlation between these two HI tests (Table 1).

Field samples

The indirect ELISA was used to determine the sero-prevalence of RVFV in domestic ruminants in different regions of South Africa for the purpose of exporting embryos of these animals. The absence of RVFV activity in South Africa over the past 14 years provided a sound basis for evaluating of the specificity of the indirect ELISA on field samples. Of a total of 10 582 field sera, 14 (0,13%) tested ELISA positive. All of 84 cattle sera tested negative, whereas 6 (0,16%) of 3 659 sheep sera and 8 (0,12%) of 6 839 goat sera tested positive (results not shown).

DISCUSSION

The fact that the ELISA detects the binding of specific antibody to RVF antigen while the VN test measures the ability of antibody to neutralize virus in an infectious system, and the HI test measures the ability of antibodies to inhibit the haemagglutinating property of the virus, suggests that differences in the level of antibody responses measured by these three serological tests should be anticipated. This fact was substantiated by the relatively poor correlation of ELISA-titre values with those of VN and HI (Table 1). Similar results were obtained in late post-vaccination and post-infection human sera tested by sandwich ELISA (Niklasson,

Peters, Grandien & Wood 1984). In spite of this, however, there was a high degree of correlation between ELISA results and those of VN and HI when compared on a positive/negative basis (Table 2). The ELISA proved to be highly sensitive and specific compared with the VN and HI tests, and the use of inactivated antigen increases its potential use in surveillance in non-endemic areas. The HI test based on hamster-liver-extracted antigen generally yielded higher titres than the HI test based on tissue-culture-derived antigen. Both were nevertheless equally capable of distinguishing between HI-positive and -negative sera. This study conclusively demonstrates the use of cell-culture-derived, acetone-extracted antigen for the detection of RVFV-specific antibodies with both the ELISA and HI tests. In addition, the method is both cost-effective and sensitive to an international trend towards minimizing the use of laboratory animals.

The low seroprevalence of RVFV antibodies in ruminants in South Africa detected by the indirect ELISA, is statistically and epidemiologically insignificant and could be attributed either to false-positive ELISA results or to the post-vaccination status of tested animals. It does, however, confirm the high specificity of the indirect ELISA when it is considered against the background of a total absence of RVFV activity in South Africa over the past 14 years.

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