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Enzyme-linked immunosorbent assays for the detection of antibody to Crimean–Congo haemorrhagic fever virus in the sera of livestock and wild vertebrates

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SUMMARY

IgM antibody response to Crimean–Congo haemorrhagic fever (CCHF) virus was monitored in experimentally infected sheep and cattle by an IgM capture enzyme-linked immunoassay (ELISA). Specific binding of antigen was detected by a rabbit anti-CCHF horseradish peroxidase conjugate or a sandwich technique with hyperimmune mouse anti-CCHF ascitic fluid and commercially available anti-mouse immunoglobulin peroxidase conjugate. The persistence of IgM antibody activity was found to be of shorter duration than in humans, and this may be a function of the relative lack of susceptibility of these animals to infection with CCHF virus. IgG antibody responses in the sheep could be monitored by sandwich ELISA using commercially available anti-sheep immunoglobulin peroxidase conjugates. Total antibody activity in the sera of experimentally infected sheep, cattle and small mammals could be monitored in a competitive ELISA (CELISA) using rabbit anti-CCHF peroxidase conjugate. The CELISA was applied to the sera of 960 wild vertebrates from a nature reserve in South Africa, and the prevalence of antibody was found to be greatest in large mammals such as rhinoceros, giraffe and buffalo, which are known to be the preferred hosts of the adult tick (*Hyalomma*) vectors of the virus.

INTRODUCTION

Crimean–Congo haemorrhagic fever (CCHF) is a zoonosis caused by a tick-borne virus which has assumed increasing importance in Africa in recent years, probably as a result of greater awareness and improved surveillance [1–8]. Although techniques such as immunodiffusion, complement-fixation, indirect immunofluorescence (IF), haemagglutination-inhibition, passive haemagglutination-inhibition and virus neutralization have been used successfully to investigate the disease, epidemiological studies have been hampered by lack of sensitivity, reproducibility and amenity to automation of the techniques [9–19]. The problems were potentially overcome by the development of a solid-phase radioimmunoassay and an indirect or sandwich enzyme-linked immunoassay (ELISA) [20], and the

ELISA has found application for the demonstration of antibody in human sera [21].

Anti-species immunoglobulin conjugates for IF and ELISA tests on sera of domestic animals are commercially available, and some may be used on the sera of wild vertebrates; e.g. anti-dog and anti-cat conjugates can be used on the sera of wild canids and felids. We developed sandwich and IgM-capture antibody ELISA for sheep and cattle sera using commercially available anti-immunoglobulins and an anti-CCHF horseradish peroxidase (HRPO) conjugate prepared from immunized rabbit serum. However, we found that sandwich techniques could not be applied successfully to the sera of all African antelope species; anti-sheep and anti-cattle immunoglobulins bound poorly or not at all to the immunoglobulins of some antelope in ELISA or immunodiffusion tests (unpublished observations). Moreover, anti-dog immunoglobulin failed to react with hyenid (aardwolf and hyena) sera, and protein A and protein G conjugates failed to react with the sera of certain wild mammals, such as elephants, hippopotamuses and springhares. Hence, here we developed a competitive ELISA (CELISA) using the rabbit anti-CCHF conjugate. The ELISA and CELISA tests were evaluated using the sera of experimentally infected sheep, cattle and small mammals, and the CELISA was then applied to the sera of 960 wild vertebrates collected in a nature reserve.

MATERIALS AND METHODS

Antigens and antisera

Sucrose-acetone (SA) extracted antigen was prepared from mouse brain infected with South African CCHF isolate 4/81 [1], and inactivated with 0·1% beta-propriolactone (BPL) (Sigma, St Louis, USA) [22]. Cell-culture lysate antigen was prepared from Vero cells grown in 150 cm^2 flasks and infected with CCHF isolate 4/81 at a multiplicity of one tissue-culture infective dose ($TCID_{50}$) per cell. After incubation for 5 days at 37 °C the cells were harvested, washed in borate-buffered saline, pH 9·0, resuspended in 0·5 ml of 1% Triton X-100, 0·1% sodium dodecyl sulphate in borate buffer per flask, and subjected to ultrasonic disruption. After centrifugation for 10 min at 9000 g and 4 °C, the supernatant fluid was inactivated with 0·1% BPL and stored at −70 °C as antigen.

Monoclonal antibody 6E5 specific for viral nucleocapsid had been prepared against CCHF isolate 41/84 [23]. Hyperimmune mouse ascitic fluid (HMAF) was prepared from adult mice inoculated intraperitoneally (i.p.) with 0·2 ml of a 1/10 dilution of mouse brain CCHF virus suspension (isolate 4/81) in Freund's complete adjuvant, once weekly for 4 weeks. On the fifth week the mice were inoculated i.p. with 0·2 ml of a suspension of sarcoma 180 cells, and 1 week later the ascitic fluid was harvested [24].

New Zealand giant rabbits were inoculated subcutaneously with $10^{4·3}$–$10^{7·0}$ $TCID_{50}$ of CCHF virus 4/81, given a booster 1 month later, and exsanguinated after a further 2 weeks. The IgG fraction was isolated from pooled immune rabbit serum by ammonium sulphate precipitation (31%, pH 7·4), followed by affinity chromatography on a protein A-Sepharose CL-4B column according to the manufacturer's instructions (Pharmacia, Uppsala, Sweden), conjugated to HRPO

type VI (Sigma) using sodium periodate (Sigma) [25, 26], and dialysed against phosphate-buffered saline (PBS) pH 7·4 containing 0·01% merthiolate.

Test sera

A total of 63 serum samples from 3 sheep, 145 samples from 11 cattle and 69 samples from 34 small mammals were obtained from individuals that had been infected subcutaneously with $10^{1.5}$–$10^{6.5}$ $TCID_{50}$ of CCHF isolate 4/81 in studies of viraemia and tick transmission [27, 28], at intervals as indicated in the results. Sera taken from each of the sheep, cattle and small mammals before infection were used as negative controls where appropriate. Sera of 29 species of wild vertebrates were collected in the Kruger National Park, South Africa, between 1974 and 1992, for present and unrelated purposes and stored at −70 °C.

Serological tests

ELISA were performed in 96-well immunoassay microplates (Nunc, Roskilde, Denmark) and optimal working dilutions of reagents were determined by chessboard titration. Unless stated otherwise, 100 μl test volumes were used, incubations were performed for 1 h at 37 °C, plates were washed thrice with PBS containing 0·1% Tween 20 (Merck, Darmstadt, Germany) (PBST), wells were post-coated with 200 μl of PBS containing 2% bovine serum albumin (Calbiochem, La Jolla, USA), and the diluent for reagents was PBS containing 10% foetal calf serum (State Vaccine, Cape Town, South Africa).

IgM-capture ELISA using anti-CCHF HRPO conjugate. Plates were coated overnight at 4 °C with μ-chain specific anti-sheep IgM (Cappel, Organon Teknika N.V., Turnhout, Belgium) diluted 1/1000 in carbonate buffer, pH 9·6. It had previously been established that major cross-reactions occurring in the IgM of bovids render this capture antibody suitable for testing cattle as well as sheep sera [29]. After the plates were washed, sheep or cattle sera were added to the wells in doubling dilutions from 1/200 upwards. The plates were incubated, washed, and CCHF antigen, diluted 1/200, was added to the wells. After further incubation and washing, anti-CCHF HRPO conjugate, diluted 1/1000, was added to the wells and the plates were incubated. After further washing, the substrate, 2,2′-azino-di-(3-ethyl-benzthiazole-6-sulphonate) (ABTS) (Kirkgaard and Perry Laboratories, Gaithersburg, USA) was added and the plates incubated at room temperature (22 °C) for 30 min in the dark. The results were determined by reading the optical density at 402 nm on a Multiscan spectrophotometer (Flow Laboratories Inc., McLean, VA, USA). Specimens were recorded as IgM antibody positive if the absorbance in the sample wells was at least twice the value of that for control serum taken from the animals prior to infection. Titres were recorded as reciprocals of the highest dilutions of test sera giving a positive result.

IgM-capture sandwich ELISA. Plates were coated overnight with anti-sheep IgM antibody, post-coated and reacted with sheep sera as above. After washing, 1/200 CCHF antigen in diluent containing 2% normal sheep serum was added and the plates incubated. The plates were washed and anti-CCHF HMAF diluted 1/1000 was added, left to react, and detected with 1/1000 anti-mouse IgG HRPO conjugate (Kirkgaard and Perry) and ABTS substrate. The results were recorded as described above. Cattle sera were not tested by this method.

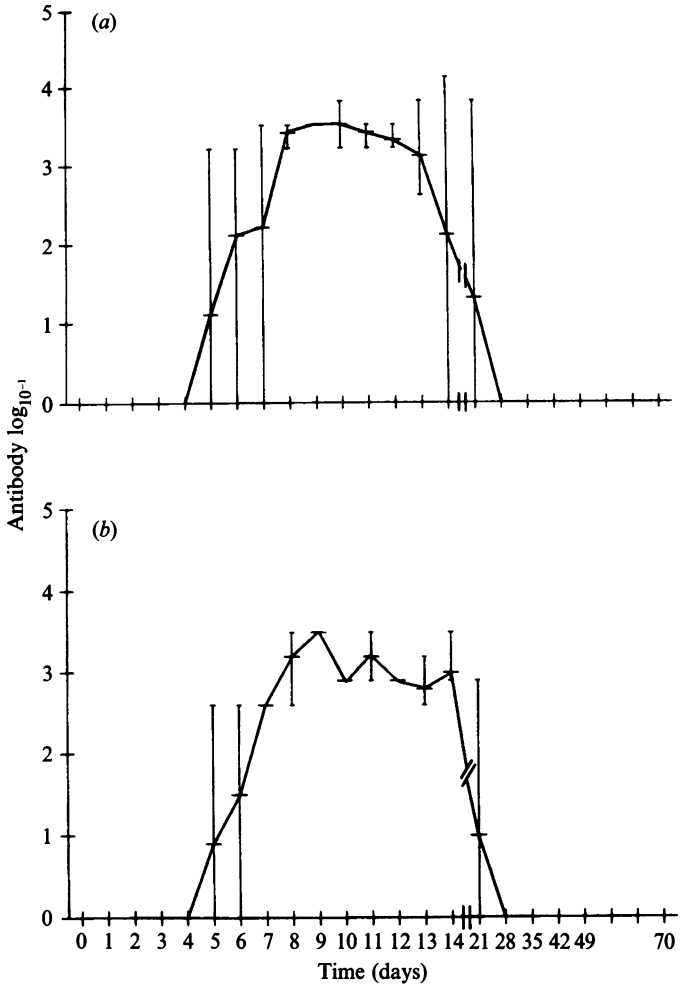

Fig. 1. IgM antibody titres against CCHF virus in sheep sera detected by IgM capture ELISA using two different detection methods: (*a*) anti-species HRPO conjugate and (*b*) anti-CCHF HRPO conjugate. Graphs show mean antibody titre plus range.

IgG sandwich ELISA. Plates were coated overnight at 4 °C with anti-CCHF monoclonal antibody diluted 1/2000. After post-coating, CCHF antigen at a dilution of 1/200 was added, the plates were incubated and washed, and sheep sera were added in doubling dilutions from 1/100 upwards. The plates were incubated, washed and anti-sheep IgG HRPO (Zymed Laboratories Inc., San Francisco, USA), was added at a dilution of 1/1000. The peroxidase was detected with ABTS substrate as above. Cattle sera were not tested by this method.

CELISA. For the CELISA [30], plates were coated overnight at 4 °C with monoclonal antibody diluted 1/7000. After post-coating, 50 μl of test serum was added to the wells in doubling dilutions from 1/10 upwards, along with 50 μl of CCHF antigen diluted 1/100. The plates were incubated for 3 h at 37 °C with gentle mixing at 30 min intervals. After washing, anti-CCHF HRPO conjugate diluted 1/1000 was added to the wells, left to react as described above and detected with ABTS substrate. The sera were considered positive if the optical

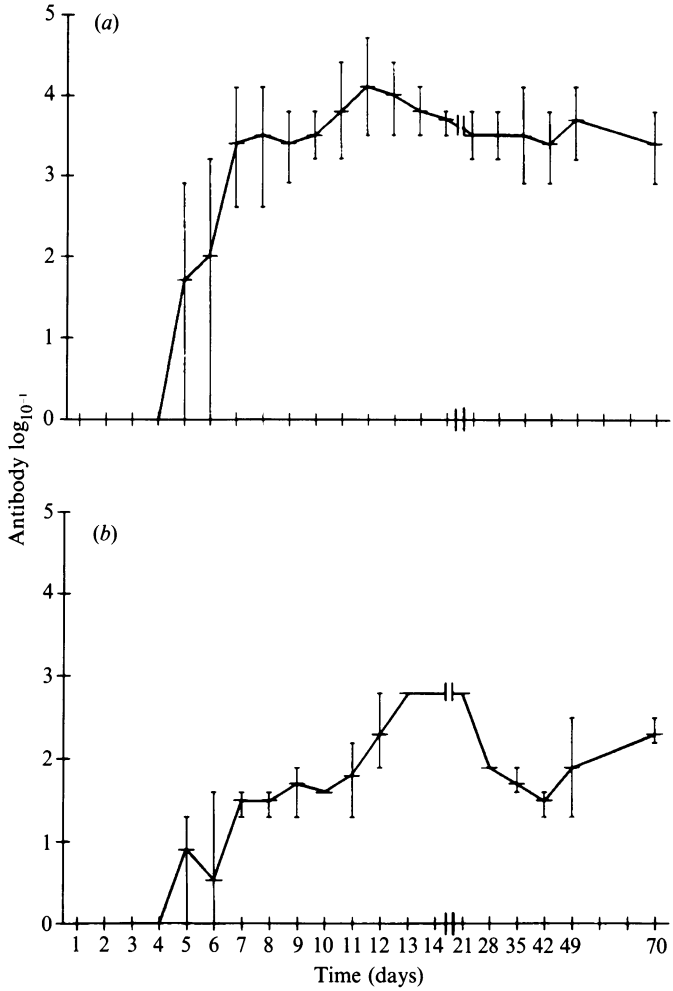

Fig. 2. Antibody response to CCHF virus in sheep sera detected by (*a*) sandwich ELISA for IgG antibody using anti-sheep HRPO conjugate and (*b*) CELISA for total antibody activity. Graphs show mean antibody titre plus range.

density reading was $\leqslant 50\%$ that for control serum taken from the animals prior to infection. An attempt was made to perform the test as a blocking rather than as a competitive assay by adding antigen and test sera sequentially instead of simultaneously.

IF tests. IF tests for the detection of IgG antibody to CCHF virus in the sera of small mammals were performed as described previously [18] using commercially available anti-mouse, -rabbit and -guinea pig immunoglobulin FITC conjugates (Cappel).

RESULTS

Detection of antibody in sheep sera

The results obtained in IgM-capture ELISAs on sheep sera are shown in Fig. 1. IgM antibody to CCHF virus was detectable from days 5–21 after infection. The method using the anti-CCHF conjugate (Fig. 1*b*) demonstrated slightly lower

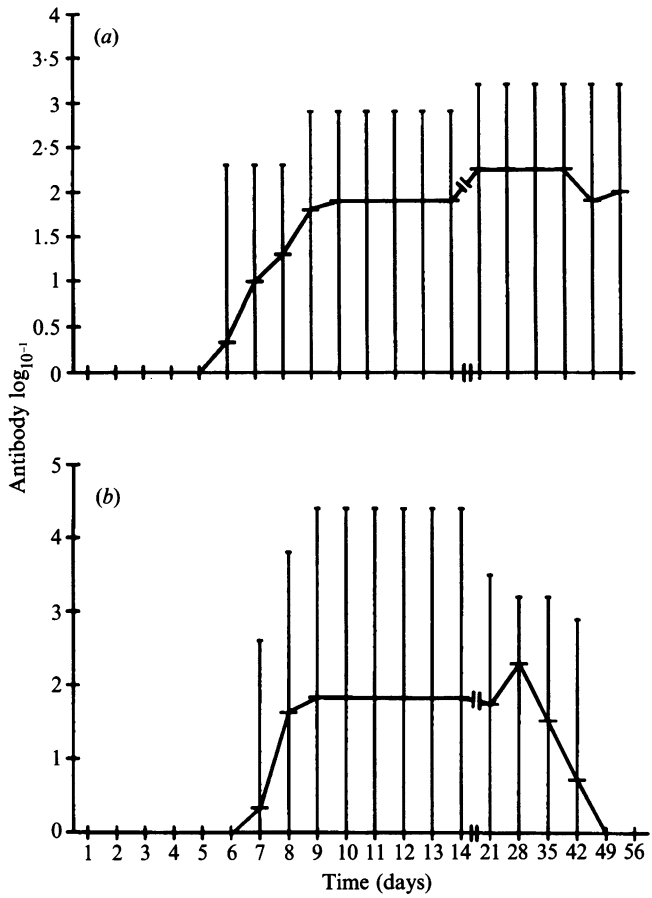

Fig. 3. Antibody response to CCHF virus in cattle sera detected by (*a*) CELISA for total antibody activity and (*b*) IgM capture antibody ELISA. Graphs show mean antibody titre plus range.

titres than that using the HMAF detection system (Fig. 1*a*), but detected antibody more frequently in sera taken early and late in the course of the response than the HMAF system.

The sandwich ELISA detected IgG antibody activity to CCHF virus in sheep sera by day 4 post-infection at the earliest (Fig. 2*a*), while the CELISA, which does not discriminate between IgG and IgM antibody, demonstrated antibody by day 5 at the earliest (Fig. 2*b*). Antibody was still demonstrable by both methods on day 70 in all three sheep, but the titres determined by the sandwich ELISA were slightly higher than those measured by the CELISA. Significantly lower titres were obtained if the CELISA was performed as a blocking test by adding antigen and test sera sequentially rather than simultaneously, and hence this option was discarded.

Detection of antibody in cattle sera

The results obtained in the capture ELISA for IgM and CELISA for total antibody activity on the sera of eleven cattle are shown in Figure 3. IgM antibody

Table 1. *Detection of antibody to CCHF virus in experimentally infected small mammals by IF and CELISA*

Species	No.	Day after inoculation	Antibody titre or range	
			ELISA	IF
Cavia porcellus (Guinea-pig)	9	21	160–320	5120
Tatera brantsii (Bushveld gerbil)	2	1	—	—
	2	21	160	640
	2	35	20–40	—
Tatera leucogaster (Highveld gerbil)	4	1	—	—
	3	28	0–320	80–640
Mystromys albicaudatus (White-tailed rat)	3	1	—	—
	3	14	0–80	0–640
	3	21	20–160	640
	3	28	20–40	160–640
Aethomys chrysophilus (Red veld rat)	2	1	—	—
	2	14	80	160–640
Xerus inauris (Cape ground squirrel)	2	43	160	640
Lepus saxatilis (Scrub hare)	4	1	—	—
	2	2–6	—	—
	2	7	10–80	40
	3	14	20–320	640
	2	21	320	320–640
	2	27	160	320–640
	2	35	80	160–320
	3	45	20–40	0
Oryctolagus cuniculi (New Zealand giant rabbit)	8	28	0–160	0–640

was detectable from day 7, but five of the cattle failed to develop demonstrable IgM response (Fig. 3 *b*). Total antibody response was detected from day 6 by CELISA (Fig. 3 *a*) and was still detectable on day 56, whereas IgM antibody titres declined after day 28 and were no longer demonstrable by day 49. Three cattle failed to develop any demonstrable antibody response. Mouse brain and cell culture lysate antigens produced similar results in all ELISA and CELISA tests.

Detection of antibody in experimentally infected small mammals

Antibody titres detected by CELISA in the sera of 34 experimentally infected small mammals (Table 1) were lower than, but bore a consistent relationship to, those detected by IF (correlation coefficient 0·8). The only exceptions were two bushveld gerbils and three hares which had low antibody titres by ELISA on days 35 and 45 post-infection respectively but were negative by IF, and one highveld gerbil which was negative by ELISA but had a low IF antibody titre on day 28.

Detection of antibody in sera of wild vertebrates

The numbers of wild vertebrate sera from the Kruger National Park that were found to have antibody to CCHF virus demonstrable by CELISA are shown in Table 2. The highest prevalences of antibody were found in some of the larger mammals such as buffalo, white rhinoceroses and giraffe. Contrary to previous findings, no antibody was found in any of the 63 hares that were screened.

Table 2. *Prevalence of antibody to CCHF virus in wild vertebrate sera collected in the Kruger National Park*

Common name	Scientific name	No. positive/no. tested (%)
Lion	*Panthera leo*	0/116 (0)
Cheetah	*Acinonyx jubatus*	0/14 (0)
Leopard	*Panthera pardus*	0/6 (0)
Genet	*Genetta genetta*	0/1 (0)
Wild dog	*Lycaon pictus*	3/62 (5)
Elephant	*Loxodonta africana*	0/23 (0)
Black rhino	*Diceros bicornis*	2/5 (40)
White rhino	*Ceratotherium simum*	21/31 (68)
Zebra	*Equus burchelli*	2/28 (7)
Warthog	*Phacochoerus aethiopicus*	0/21 (0)
Hippopotamus	*Hippopotamus amphibius*	0/15 (0)
Giraffe	*Giraffa camelopardalis*	10/44 (23)
Impala	*Aepyceros melampus*	5/47 (11)
Blue wildebeest	*Connochaetes taurinus*	0/31 (0)
Tsessebe	*Damaliscus lunatus*	0/2 (0)
Buffalo	*Syncerus caffer*	31/312 (10)
Nyala	*Tragelaphus angasii*	0/1 (0)
Bushbuck	*Tragelaphus scriptus*	0/1 (0)
Kudu	*Tragelaphus strepsiceros*	2/4 (50)
Duiker	*Sylvicapra grimmia*	0/1 (0)
Roan	*Hippotragus equinus*	0/8 (0)
Sable	*Hippotragus niger*	3/49 (6)
Hartebeest	*Sigmoceros lichtensteinii*	0/1 (0)
Suni	*Neotragus moschatus*	0/4 (0)
Baboon	*Papio ursinus*	0/21 (0)
Vervet monkey	*Cercopithecus pygerythrus*	0/1 (0)
Porcupine	*Hystrix africaeaustralis*	0/2 (0)
Hare	*Lepus saxatilis*	0/63 (0)
Guinea fowl	*Numida meleagris*	2/37 (5)
Ostrich	*Struthio camelus*	0/9 (0)

DISCUSSION

These results indicate that IgM antibody to CCHF virus can be demonstrated in sheep sera by IgM-capture ELISA with either of two detection methods, using rabbit anti-CCHF conjugate or a sandwich technique with anti-CCHF serum and a commercial anti-species immunoglobulin conjugate. Titres detected with the anti-CCHF conjugate were slightly but not significantly lower than those detected by the sandwich method. Furthermore, it was confirmed that the technique using anti-sheep IgM-capture antibody can also be used on cattle sera, as has been demonstrated previously with antibody to Rift Valley fever virus [29]. That IgM antibody titres in sheep and cattle sera only remained demonstrable for up to 3–7 weeks respectively following infection is in contrast to the 3–5 months recorded in human patients [21], and is probably a function of the relative lack of susceptibility of these animals to infection with the virus. The experimental infections in the sheep and cattle produced only transient low-titre viraemia and mild fever [31]. In contrast, the virus is highly pathogenic to humans, causing illness with 30% mortality. In guinea fowl, of which only a proportion of individuals develop low-grade viraemia or demonstrable antibody response

following infection, total antibody titres decline to undetectable levels in about 7 weeks, while domestic chickens fail to develop either demonstrable viraemia or an antibody reponse [32].

Total antibody titre or IgG antibody in sheep sera can be demonstrated by CELISA or a sandwich method using commercial anti-species conjugate. The CELISA can also be used to detect antibody in the sera of cattle and small and large wild vertebrates, and therefore has an important application in epidemiological surveys for antibodies to many agents, including Marburg and Ebola haemorrhagic fever viruses, for which the natural hosts are unknown.

The prevalence of antibody to CCHF virus detected in sera from the Kruger National Park is lower than was found previously in wild vertebrates from other locations in southern Africa [1, 19], and this may be related to the fact that *H. truncatum* is the only species of *Hyalomma* believed to occur in the Park [33–37]. *Hyalomma* ticks are considered to be the principal vectors of CCHF virus, and it had been observed earlier [4] that there was a much lower prevalence of antibody to the virus in cattle sera in the south-western portion of the Cape province of South Africa, where *H. truncatum* is also the only representative of the genus, than elsewhere in southern Africa. The fact that the highest prevalences of antibody occurred in the sera of animals such as the rhinoceros, giraffe and buffalo in the Kruger National Park is in accordance with the previous observation that CCHF infection appears to occur most frequently in larger mammals, which are the preferred hosts of adult *Hyalomma* ticks [19]. Immature *Hyalommas* feed on ground-frequenting birds and small mammals, and although antibody was found in two guinea-fowl sera (Table 2), the lack of CCHF antibody in hares in the Kruger National Park is in contrast to the situation observed elsewhere in the subcontinent [1, 19]. The larger mammals have much longer lifespans than hares, and this would be conducive to the occurrence of greater cumulative prevalences of antibody, particularly in locations where the challenge rate is inherently low.

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