11-1994

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Am. J. Trop. Med. Hyg., 51(5), 1994, pp. 568–576

EVIDENCE OF NATURAL BLUETONGUE VIRUS INFECTION AMONG AFRICAN CARNIVORES

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Abstract. Bluetongue is an International Office of Epizootics List A disease described as the century's most economically devastating affliction of sheep. Bluetongue (BLU) viruses were thought to infect only ruminants, shrews, and some rodents, but recently, inadvertent administration of BLU virus–contaminated vaccine resulted in mortality and abortion among domestic dogs. We present evidence of natural BLU virus infection among African carnivores that dramatically widens the spectrum of susceptible hosts. We hypothesize that such infection occurred after ingestion of meat and organs from BLU virus–infected prey species. The effect of BLU virus on endangered carnivores such as the cheetah and African wild dog requires urgent investigation. Also, the role of carnivores in the epizootiology of this disease needs elucidation.

The bluetongue (BLU) serogroup of orbiviruses are insect-transmitted and infect wild and domestic ruminants, principally sheep. The disease is common in tropical, subtropical, and some temperate regions of the world. Twenty-five different BLU virus serotypes are currently identified.[1] Bluetongue was first recognized when susceptible European sheep breeds were introduced into South Africa in the 17th century. It consequently was proposed that BLU virus originated in Africa and was spread to other parts of the world,[2] although recent genetic analyses indicate that several BLU virus serotypes could have had a long evolutionary history in North America.[3]

The consequences of BLU virus infection differ among ruminant hosts. Sheep infected with the viruses may show signs of bluetongue disease, whereas infection of cattle is typically asymptomatic.[4] Very little is known about the effect of BLU virus infection on wild ungulates in Africa; serologic studies indicate that a large proportion of wild ruminant populations have been infected by the viruses,[5] but little information is available on the adverse consequences of such infection. Bluetongue virus–associated mortality has been described in a free-ranging population of topi (*Damaliscus korrigum*)[6] and a captive eland (*Tragelaphus strepsiceros*).[7] Buffalo (*Syncerus caffer*) calves died after experimental infection,[8] whereas blesbok (*Damaliscus albifrons*) developed subclinical infections but had a sufficient viremia to cause clinical disease among sheep injected with blesbok blood.[9]

While a number of serologic surveys for BLU virus antibodies have been conducted among African ungulates[10,11] and small mammals,[12] no such serosurveys have been reported for carnivores. A related group of viruses, African horse sickness (AHS), have long been known to infect carnivores.[13] Domestic dogs are susceptible and have been reported to be capable of infecting the tick vectors of AHS.[13] Domestic dog mortality and abortion were recently documented following inadvertent administration of a BLU virus–contaminated vaccine.[14] We report here that natural occurrence of antibodies to BLU virus is widespread among African carnivores.

MATERIALS AND METHODS

Samples used in this study were largely collected in conjunction with our carnivore disease

and genetic surveys and were stored at −20°C and −80°C in various serum banks until tested. Serum samples were collected from African carnivores including domestic dogs (*Canis familiaris,* Moremi, Botswana; Masai Mara, Kenya), jackals (*Canis* spp., various locations in Kenya), African wild dogs (*Lycaon pictus,* Moremi, Botswana; Masai Mara, Kenya; Kruger National Park, Republic of South Africa; Serengeti National Park, Tanzania; Chipangali Breeding Centre, Zimbabwe), Simien wolves (*Canis simiensis,* Bale Mountains National Park, Ethiopia), spotted hyenas (*Crocuta crocuta,* Masai Mara, Kenya), domestic cats (*Felis catus,* Masai Mara, Kenya), cheetahs (*Acinonyx jubatus,* Kruger National Park, Republic of South Africa; Serengeti National Park, Tanzania), lions (*Panthera leo,* Ngorongoro Crater and Serengeti National Park, Tanzania; Kruger National Park, Republic of South Africa), large-spotted genets (*Genetta maculata,* Masai Mara, Kenya), white-tailed mongooses (*Ichneumia albicauda,* Masai Mara, Kenya), and marsh mongooses (*Atilax paludinosus,* Masai Mara, Kenya). Serum samples tested from American carnivores included coyotes (*Canis latrans,* California) and domestic dogs (Yolo and Lancaster County Pounds, California). Samples from captive African wild dogs in America were made available by the Brookfield, Cheyenne Mountain, and San Diego Zoos.

Serum antibodies specific for a viral core protein (VP-7) of BLU virus were identified with a commercial competitive enzyme-linked immunosorbent assay (cELISA, Blueplate Special; Diagxotics Inc., Wilton, CT) and samples were considered positive if they showed inhibition that was ≥ 30% of the negative control.[15] This test is highly sensitive and specific for the bluetongue serogroup of orbiviruses (98% and 99.4%, respectively) and has been tested for cross-reactivity with a variety of related and nonrelated viruses including AHS serotypes 1-9, epizootic hemorrhagic disease virus, bovine herpesvirus 1-3, bovine parvovirus, respiratory syncytium virus, bovine leukosis virus, bovine viral diarrhea, parainfluenza-3 virus, and anaplasma, *Brucella abortus,* and *Mycobacterium paratuberculosis* (Reddington JJ, unpublished data).

Sera were tested for neutralizing antibody against BLU viral serotypes 1-20 using a microtiter virus neutralization assay.[16] Cell culture–adapted BLU viral serotypes were titrated and diluted to obtain a virus concentration between 100 and 400 50% tissue culture infective dose ($TCID_{50}$) units. Titers were expressed as the inverse of the final dilution of serum that provided at least 50% protection of Vero cell monolayers. Serum titers > 30 were considered positive, and samples were not tested beyond a dilution of 1:120.

For the immunoprecipitation assay,[17] a confluent monolayer of BHK-21 cells was infected with BLU-10 at a multiplicity of infection of 0.5. Twelve hours after infection, ^{35}S-methionine (100 μCi/ml) was added in methionine-deficient minimal essential medium. Cells were labeled for 1 hr, then washed three times with phosphate-buffered saline before extracting with NET-NP40 (0.15M NaCl, 50 mM Tris, pH 8.0, 40 mM EDTA, 0.01% NaN_3, 1 mM phenylmethylsulfonyl fluoride, and 0.05% NP-40) for 5 min. Nuclei were removed by centrifugation at 400 × *g* for 5 min. The ^{35}S-methionine–labeled lysate was preadsorbed with a 1% volume of washed Pansorbin (*Staphylococcus aureus;* CalBiochem, La Jolla, CA) for 1 hr. The lysate was then centrifuged at 72,000 × *g* for 1 hr. The preadsorbed, virion-free preparation of ^{35}S-methionine–labeled BLU viral proteins was incubated with 25 μl of antisera for 1 hr before adding 50 μl of 25% Protein A agarose beads. Ten micrograms of rabbit anti-canine IgG was added to all dog and hyena sera to facilitate binding to Protein A beads. After an additional 30-min incubation, the reaction was washed three times with NET-NP40. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis sample buffer was added and the samples were subjected to electrophoresis and autoradiography. Identical immunoprecipitations were performed with mock-infected BHK-21 cells as a negative control.

Seroprevalence levels among species and locality were compared by chi-square analyses; in comparisons where cells contained values of 5 or less, Fisher's exact test was used.[18]

RESULTS

Seroprevalence of BLU viral antibodies. Seroprevalence of antibodies to BLU virus by species and location is presented in Table 1. Positive individuals were identified among most of the free-ranging African carnivores sampled (cheetahs, lions, domestic cats, African wild dogs, jackals, domestic dogs, hyenas, large-spotted genets). Variable levels of seroprevalence

TABLE 1

*Seroprevalence of bluetongue virus–specific antibodies among nonruminant species**

Nondomestic carnivores in Africa							
Species	Botswana	Ethiopia	Kenya	Namibia	South Africa	Tanzania	Zimbabwe
Wild dog	23/24 (96)	NP	6/18 (33)	NS	10/12 (83)	8/18 (44)	0/7 (0)†
Jackal	1/3 (33)	NS	3/56 (5)	NS	NS	NS	0/9 (0)†
Simien wolves	NP	0/25 (0)	NP	NP	NP	NP	NP
Cheetah	NS	NS	NS	NS	0/2 (0)	Ng 0/2 (0) Se 4/33 (12)	NS
Lion	NS	NS	NS	19/36 (53)	29/36 (81)	Ng 28/32 (88) Se 15/33 (46)	NS
Spotted hyena	NS	NS	33/60 (55)‡ 33/76 (43)§	NS	NS	NS	NS
W.t. mongoose	NS	NS	0/15 (0)	NP	NP	NS	NS
M. mongoose	NS	NS	0/5 (0)	NS	NS	NS	NS
Spotted genet	NS	NS	2/10 (20)	NS	NS	NS	NS

Domestic carnivores in Africa			
Species	Botswana	Ethiopia	Kenya
Dog	2/92: 13/41 (32) 11/92: 20/52 (39)	1990: 0/7 (0)	7/89: 9/51 (18) 7/90: 20/87 (23) 8/91: 4/53 (8) 10/92: 13/63 (22)
Cat	NS	NS	10/92: 7/34 (21)

Domestic and nondomestic carnivores in the United States	
Species	United States
Domestic dog	0/174 (0)
Coyote	0/10 (0)
African wild dog	0/13 (0)†

* Values are the no. positive/no. tested (%). Carnivore sampling localities were Botswana–Moremi; Kenya–Masai Mara, jackals were sampled in several localities; Tanzania–Serengeti National Park (Se) and Ngorongoro Crater (Ng); Ethiopia–Bale Mountains National Park; Zimbabwe–Bulawayo; South Africa–Kruger National Park; United States–coyotes from California, domestic dogs from Yolo and Lancaster County (CA) pounds, captive wild dogs from zoos. NP = species not present in this locality; NS = species not sampled.

† Captive population.

‡ Hyena population sampled in 1980.

§ Hyena population sampled in 1990.

were noted among species: jackals and cheetahs characteristically had low numbers of seropositive individuals, while most lions and hyenas tested positive for BLU viral antibodies. Positive individuals often had high percent inhibition values (Figure 1).

No seropositive individuals were detected among free-ranging Ethiopian wolves, marsh mongooses, or white-tailed mongooses. All captive African wild dogs from both Zimbabwe and the United States were seronegative. Coyotes and domestic dogs from California were tested to determine the route of infection (i.e., oral versus vector).

Immunoprecipitation. An immunoprecipitation assay was used to confirm specific reaction of individual sera to the core protein (VP-7) of BLU virus (Figure 2). Seropositive samples strongly precipitated soluble VP-7, whereas seronegative samples did not precipitate any viral protein.

Serum neutralization. Neutralizing antibodies to 12 of 20 BLU viral serotypes were identified in carnivore sera, and antibodies to BLU viral serotypes 3, 8, 13, and 17 were especially prevalent (Table 2). Neutralizing antibodies to BLU viral serotypes 6 and 19 were exclusively identified among domestic cats from the Masai Mara, Kenya. Serotype 14 neutralizing antibodies were detected solely among domestic dogs and hyenas from this same area. Only African wild dogs from Kruger National Park in the Republic of South Africa demonstrated neutralizing antibodies to BLU viral serotypes 2 and 18. More than 50% of the samples positive for neutralizing antibody had titers greater than or equal to 120. Of the 163 cELISA-positive samples, however, 77 did not demonstrate the presence of

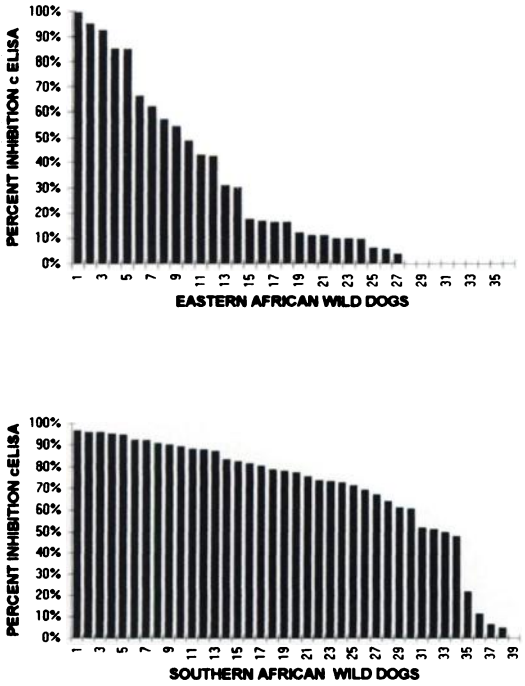

FIGURE 1. Comparison of competitive enzyme-linked immunosorbent assay (cELISA) percent inhibition among wild dogs sampled in eastern Africa (Kenya and Tanzania) and southern Africa (Botswana and the Republic of South Africa). Significant differences in bluetongue virus seroprevalence were found between these groups; also, southern African wild dogs had considerably higher levels of inhibition compared with eastern African wild dogs.

neutralizing antibodies against BLU viral serotypes 1-20 with the test criteria used.

DISCUSSION

Antibodies to BLU viruses are prevalent among a variety of free-ranging African herbivore species (Table 3). Tests with varying specificities and sensitivities (agar gel immunodiffusion,[10, 19-22] cELISA,[11] and fluorescent antibody[23, 24]) were used to screen the herbivores, but nevertheless, the surveys indicate that wild and domestic ruminants in eastern and northeastern Africa (Kenya, Tanzania, Egypt, Sudan) usually have lower BLU viral antibody seroprevalence levels than those in southern Africa (Botswana, Zambia).

Antibodies to 12 BLU viral serotypes were noted among the African carnivores tested. These serotypes have largely been described in Africa,[25] with the exception of serotype 17, which has been identified only in the United States and parts of India. While many individuals reacted with only a single serotype, some animals had neutralizing antibodies to several different BLU viral serotypes. Whereas serotypes are commonly used to define BLU virus variants, it might not be valid to classify BLU virus into such distinct groups;[25] cross-reactions between serotypes and reassortment of viral genes among cocirculating viruses have been noted.[26, 27] In addition, BLU virus nucleotide sequence analyses have indicated a greater amount of divergence between the same serotype from different geographic locations (serotype 1 from Australia and Africa) than between different serotypes from the same location (serotypes 1 and 20 from Australia).[28] We are therefore hesitant to draw specific conclusions from these data but would suggest that the data indicate exposure to an array of BLU viruses.

Of the 163 samples tested for neutralizing antibodies, 47% did not react with BLU viral serotypes 1-20. The cause of this finding was not determined, but several potential explanations might be proposed. Virus $TCID_{50}$ and sample dilution were selected to increase specificity because cross-reactions among serotypes are an acknowledged problem in BLU viral diagnostics.[25] However, this also reduces the sensitivity of the test. Furthermore, BLU virus 25, which was recently identified in Kenya,[1] could not be obtained for the neutralization assays. This serotype does not cross-react with any other BLU viral serotypes.[1] There also may be other unidentified serotypes of BLU virus that would be detected by group reactive tests such as the cELISA but not by neutralization tests with existing viruses.

Since this was a retrospective serosurvey, we did not have appropriate samples to isolate virus, but both the high cELISA and virus neutralization titers imply that BLU virus had replicated in the seropositive carnivores. The route of BLU virus infection for these carnivore species is unknown but could include either vector transmission or ingestion. Vector transmission of BLU virus between ruminants is almost exclusively by *Culicoides* midges,[29] but oral infection of domestic dogs has previously been described for a closely related group of viruses that cause AHS.[30] Experimental oral infection of sheep with BLU virus resulted in clinical disease,[31] and

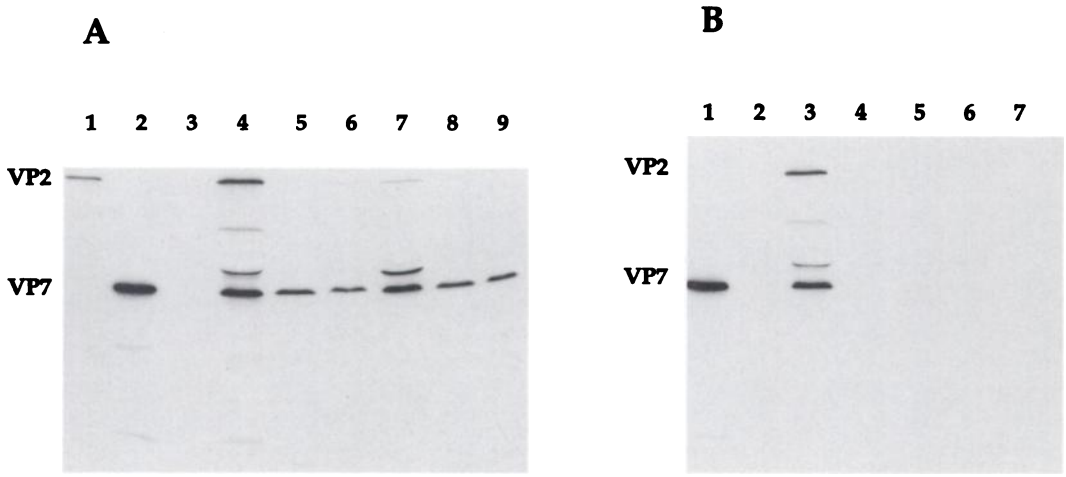

FIGURE 2. Immunoprecipitation of ^{35}S-methionine–labeled bluetongue virus-10 (BLU-10) proteins. **A,** lane 1, monoclonal antibody (MAb) 034 α BLU viral core protein-2 (VP-2); lane 2, MAb 290 α BLU VP-7; lane 3, normal rabbit sera (NRS); lane 4, polyclonal rabbit α BLU-10 (#1097, 12/15/89); lane 5, hyena (positive by competitive enzyme-linked immunosorbent assay [cELISA]: +); lane 6, lion (+); lane 7, domestic dog (+); lane 8, domestic cat (+); lane 9, cheetah (+). **B,** lane 1, MAb 290; lane 2, NRS; lane 3, rabbit α BLU 10; lane 4, hyena (negative on cELISA: −); lane 5, lion (−); lane 6, domestic dog (−); lane 7, captive wild dog from the United States (−). Viral proteins are identified at the left of each autoradiograph. Note the precipitation of VP7 by seropositive carnivore sera.

we suspect that infection among carnivores occurred by this route. We base this statement on observed seroprevalence levels among and geographic trends within carnivore species. For example, the highest seroprevalence levels were noted among large carnivores such as lions and hyenas that prey on large ruminant species with a high percentage of seropositive individuals, including buffalo, wildebeest, and cattle. Carnivores such as African wild dogs prey mainly on smaller herbivores such as impalas and Thomson's gazelles.[32] These gazelles had significantly lower BLU viral seroprevalence levels ($P < 0.01$, $\chi^2 = 64.47$) than other bovid species.[24]

TABLE 2

*Distribution of bluetongue virus serotypes 1–20 among African carnivores**

Species	Serotypes											
	2	3	4	6	8	9	10	13	14	17	18	19
Eastern Africa												
Domestic dog (K)		X					X	X	***X***			
Domestic cat (K)				***X***								***X***
Jackal (K)		X										
Wild dog (K)					X			X				
Hyena (K)		X	X		X	X		X	***X***	X		
Lion (T)		X			X					X		
Cheetah (T)					X			X		X		
Wild dog (T)					X	X				X		
Southern Africa												
Domestic dog (B)		X	X		X					X		
Wild dog (B)		X			X	X		X		X		
Lion (SA)		X			X		X			X		
Wild dog (SA)	***X***	X	X		X		X	X		X	***X***	

* K = Kenya; X = serotype present among individuals tested; ***X*** = serotype unique to species or region; T = Tanzania; B = Botswana; SA = South Africa.

TABLE 3

*Seroprevalence of bluetongue virus–specific antibodies among African ruminants (herbivores) compiled from published surveys**

Herbivore†	Botswana[10,31]	Egypt[29]	Kenya[30,33]	Sudan[28]	Tanzania[11]	Zambia[27]
Buffalo	283/325 (87)	NS	7/19 (37)	NS	329/359 (92)	NS
Gazelle	NP	NP	22/151 (7)	NP	NS	NP
Impala	19/23 (83)	NS	36/114 (32)	NS	NS	NS
Topi/tsessebe	10/13 (77)	NP	11/38 (29)	NS	3/22 (14)	NS
Hartebeest	2/3 (67)	NP	79/104 (76)	NP	10/12 (83)	NP
Wildebeest	5/13 (38)	NP	81/134 (60)	NP	45/98 (46)	NS
Cattle	548/583 (94)	5/30 (17)	289/634 (46)	70/874 (8)	NS	187/214 (87)
Sheep	137/381 (36)	425/2,933 (14)	239/938 (25)C	276/980 (28)	NS	NS
Goat	230/278 (83)	13/110 (12)	C	11/98 (11)	NS	NS
Camel	26/32 (81)	4/31 (13)	NS	5/102 (5)	NS	NS

* Values are the no. positive/no. tested (%). NS = species not sampled; NP = species not present at this locality; C = goats and sheep were combined in Kenya.

† Buffalo (*Syncerus caffer*); gazelle (*Gazella thomsoni*); impala (*Aepyceros melampus*); topi (*Damaliscus lunatus*); hartebeest (*Alcelaphus buselaphus*); wildebeest (*Connochaetes taurinus*).

Thomson's gazelles are not found in southern Africa, but impalas had a significantly lower seroprevalence of BLU viral antibodies in Kenya than in Botswana (32% and 83%, respectively; $P < 0.01$, $\chi^2 = 20.74$).[22,24] This trend among impalas is reflected in BLU viral antibody prevalence levels and percent inhibition among populations of their eastern and southern African wild dog predators (33% and 96%, respectively; $P < 0.01$, $\chi^2 = 21.38$; Figure 2).

Carnivore species such as white-tailed mongooses, large-spotted genets, and jackals that largely scavenge from herbivore carcasses were either seronegative or had low seroprevalence levels. This is compatible with their scavenging of meat and skin rather than ingestion of organs such as spleen and liver, which are rapidly consumed by the predators that killed the prey. Spleen contains the highest concentration of virus-infected blood in BLU virus–infected sheep and cattle.[33] Marsh mongooses, which primarily prey on aquatic invertebrates,[34] were seronegative despite living in close sympatry with infected ungulates.

Captive wild dogs and jackals in Zimbabwe were largely fed horse meat and therefore, oral infection with BLU virus would be unlikely.[35] However, captive canids would likely have been exposed to the vector because they were kept by open paddocks containing wild ruminant species. All captive wild dogs and jackals were seronegative, which further suggests that natural BLU virus infection of carnivores occurs orally.

Ethiopian wolves and domestic dogs sampled from the Bale Mountains National Park in Ethiopia were all seronegative. This high altitude area (> 3,000 m above sea level[36]) does not support *Culicoides* midges,[37] and therefore, viremic ruminants would not be expected in this region.

African domestic dogs may have been infected after ingestion of livestock or wildlife offal, which forms a consistent part of their diet. Like wild dogs, domestic dogs in Kenya had significantly lower seroprevalence levels than dogs in Botswana (22% and 39%, respectively; $P = 0.03$, $\chi^2 = 4.42$). In Kenya, domestic cats lived together with domestic dogs in Maasai tribal households and were sampled simultaneously. Among all the carnivores examined, however, domestic cats alone tested positive for BLU virus 6 and 19 neutralizing antibodies (Table 2). These serotypes have previously been recorded among sheep in Africa[25] and the Middle East.[38] Kenyan domestic dogs and cats from the same household thus had been exposed to different BLU viruses, and we propose this is best explained by differences in diets.

In this study, domestic dogs sampled from BLU virus–endemic regions of the United States such as California were negative for BLU viral antibodies. This observation is rather surprising given the results obtained from African domestic dogs. However, domestic dogs in the United States are generally fed commercial diets and would be unlikely to have access to uncooked meat from viremic ruminants. A survey recently conducted among domestic dogs in other parts of the United States identified a number of seropositive animals; the BLU virus–contaminated canine vaccine was implicated as the cause of seroconversion in these dogs (Osborne BI, MacLachlan NJ, unpublished results). If vectors

had been important in transmission of BLU viruses to domestic dogs, more seropositive individuals would have been expected.

Despite these observations, variation in host preference among vectors cannot be excluded as an alternative explanation for the differences noted in seroprevalence levels. *Culicoides* species have been shown to exhibit preferences among ruminant and avian species,[39] and viral serotype has been shown to influence the rate of infection of *C. variipennis*.[40] Carnivores such as lions and hyenas, which exhibit a high seroprevalence of BLU viral antibody, might thus be preferred hosts over species such as jackals and mongooses. Similarly, vectors that prefer domestic cats might be more competent in transmitting serotypes 6 and 19 than vectors that prefer domestic dogs. Studies that include host identification from blood meals,[39] virus isolation, and taxonomic assessment of *Culicoides* could be used to determine the applicability of such alternative explanations.

Carnivores had stable antibody prevalence levels over time. No significant differences were detected in seroprevalence of BLU viral antibodies between sampling periods among Botswanan and Kenyan domestic dogs. Similarly, no significant differences were found among hyena samples from the same clan collected 10 years apart (Table 1).

Two important questions emerge from this study. The first concerns the potential impact of BLU virus infection on susceptible carnivore hosts and the importance of this infection in carnivore population structure and regulation. Bluetongue viruses are pathogenic for pregnant domestic dogs, and BLU virus infection may therefore have serious consequences for endangered wild relatives. In addition, infection could manifest itself in a more insidious fashion through decreased competitive fitness, reproductive success, survival, and dispersal capability. The second question concerns the role of carnivores in the epizootiology of BLU virus infection. Outbreaks of BLU virus unexplained by importation of domestic or wild ruminants have occurred in Portugal and Cyprus, for example. These outbreaks have been attributed to wind dispersion of infected vectors,[41,42] but an alternative explanation is that these outbreaks resulted from transport of infected carnivores such as domestic dogs. It is therefore important to determine whether BLU virus can be vector transmitted from carnivores to ruminants or whether carnivores are dead-end hosts that only acquire infection after ingestion of BLU virus–infected tissues from herbivores.

Acknowledgments: We thank Paul Rossito for laboratory assistance, Itsuro Yamani for samples of domestic dogs, and Claudio Sillero-Zubiri and Dada Gotelli for serum samples from Ethiopian wolves. Brookfield, Cheyenne Mountain, and San Diego Zoos and the Chipangali Breeding Center contributed samples of carnivores in their collections.

Financial support: The Seebe Trust, Wildlife Concern International, and the Hanley Foundation granted partial financial support, and Diagxotics, Inc. provided technical and material support.

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