RETROSPECTIVE EVALUATION OF LEPTOSPIROSIS IN FREE-RANGING AND CAPTIVE BLACK RHINOCEROSES (*DICEROS BICORNIS*) BY MICROSCOPIC AGGLUTINATION TITERS AND FLUORESCENT ANTIBODY TESTING

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Abstract: To determine exposure of wild and captive black rhinoceroses (*Diceros bicornis*) to *Leptospira interrogans* and to investigate the role of leptospirosis in cases of hemolytic anemia seen in captive black rhinoceroses, serum samples from 63 free-ranging and 29 captive black rhinoceroses were tested for antibodies against *L. interrogans*. A microscopic agglutination test was used to detect antibodies against eight serovars. Free-ranging black rhinoceroses had low levels of antibody against various serovars of *L. interrogans* tested. The incidence of antibodies and the serovars involved varied in rhinoceroses captured in different geographic and ecological areas. Serum was tested from nonvaccinated captive rhinoceroses and from those that had been vaccinated with a pentavalent leptospiral vaccine. Leptospiral titers in nonvaccinated captive rhinoceroses were generally low, even in rhinoceroses that had survived or been exposed to mild episodes of hemolytic anemia. Postvaccination titer responses were similar to those expected in domestic species. Biannual vaccination with a leptospiral vaccine is recommended. To further investigate the role of leptospirosis in hemolytic anemia, a fluorescent antibody test was done on frozen liver tissue from eight captive black rhinoceroses (four of eight animals died of acute hemolytic anemia) to detect the presence of *L. interrogans*. Leptospires were detected in the liver of three of four black rhinoceroses that died of hemolytic anemia and in another rhinoceros that died of complications of ulcerative skin disease.

Key words: Leptospirosis, *Leptospira interrogans*, microscopic agglutination, black rhinoceros, *Diceros bicornis*, Zambezi Valley, Zimbabwe, Damaraland, Namibia.

INTRODUCTION

Infection with the spirochete bacterium *Leptospira interrogans* produces clinical disease in rodents, domestic animals, and humans.[2] In several species, clinical signs of infection may include hemolytic anemia with subsequent hemoglobinuria.[8,17] Be-

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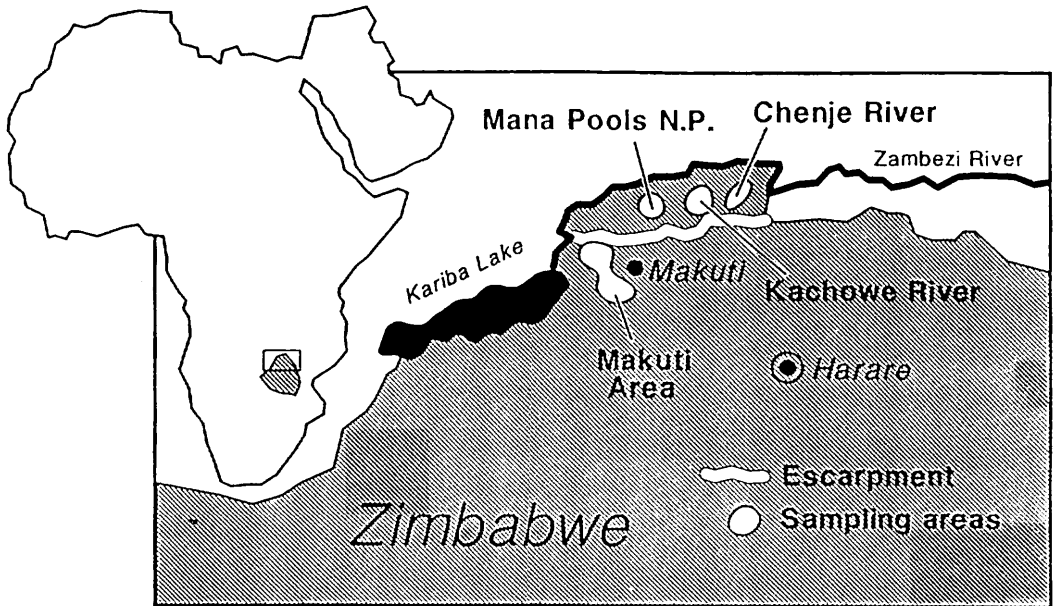
cause of the high incidence of hemolytic anemia in captive black rhinoceroses (*Diceros bicornis*), leptospirosis is considered as a possible etiology for this condition and has been implicated as a cause for the hemolysis in at least nine captive black rhinoceroses (of 31 identified cases).[3,7,9,12,13,15] However, in many of these cases, paired serum samples were not collected because of the peracute and often fatal nature of the syndrome. Although, leptospirosis has been described in a wild black rhinoceros from Kenya,[15] seroprevalence data are not available for wild black rhinoceros populations. The purpose of this study was to determine the prevalence of antibodies against *L. interrogans* in free-ranging and captive black rhinoceroses and to further investigate the role of leptospirosis in the hemolytic anemia syndrome seen in captive animals.

Figure 1. In Zimbabwe, black rhinoceroses were sampled during capture for relocation from the Zambesi River Valley in areas along the drainages of the Kachowe River, the Chenje River, and in the Mana Pools National Park. An additional group of animals was sampled in the drier and well-drained areas of the Zambesi escarpment near the town of Makuti.

MATERIALS AND METHODS

Animals and serum samples

Serum was collected from 63 wild-caught black rhinoceroses during translocation projects in Zimbabwe (*n* = 60) and Namibia (*n* = 3). Thirty-two black rhinoceroses were captured in 1988 and 1989 in areas of the Zambezi River Valley characterized by seasonally heavy rainfall, dense undergrowth, and the presence of seasonal pans and ponds (Fig. 1). Eleven rhinoceroses were captured on the Zambezi escarpment, which is savanna–woodland and has better drainage than the Zambezi River Valley. Three rhinoceroses were captured in the Damaraland of Namibia, an extremely arid area. Samples were also available from 17 other rhinoceroses captured in Zimbabwe, but the location of capture was not precisely recorded or only one or two animals were captured in those areas. None of the wild-caught rhinoceroses exhibited signs of hemolytic anemia or leptospiral infection nor had they been vaccinated.

Forty-nine serum samples were collected from 29 black rhinoceroses in captivity in North America. Thirty of the samples were from 26 nonvaccinated rhinoceroses. This group included four rhinoceroses that died of acute hemolytic anemia, four rhinoceroses that survived mild episodes of hemolytic anemia, and three rhinoceroses housed with rhinoceroses experiencing hemolytic anemia. In rhinoceroses that survived mild hemolytic anemia and in those exposed to rhinoceroses with hemolytic anemia, the average interval from disease to sampling was 37 mo (range, 12–84 mo) and from exposure to affected rhinoceroses to sampling was 27 mo (range, 12–36 mo). In addition, serum was collected from nine black rhinoceroses that had been vaccinated with a pentavalent leptospiral vaccine. Serum was obtained on an opportunistic basis from 0.5 to 24 mo after vaccination.

Serum was also obtained from seven white (*Ceratotherium simum*) and one greater Asian one-horned (*Rhinoceros unicornis*) rhinoceros in North American zoos. All se-

Table 1. Prevalence of agglutinating antibody titers ≥ 1:100 against *Leptospira interrogans* serovars in free-ranging and captive rhinoceroses.

Species climate	Location[a]	n	Serovar[b]							
			aut	brat	cani	gripp	hard	ict	pom	tar
Free-ranging black rhinoceroses										
Wet	Kachowe	16	0	6	0	4	0	15	0	7
	Mana	9	0	2	0	0	0	3	2	7
	Chenje	7	0	1	0	0	0	2	0	7
Drier, well drained	Escarpment	11	0	0	0	1	0	0	0	2
Unspecif.	Scattered	17	0	2	1	1	0	1	2	4
Arid	Namibia	3	0	0	0	0	0	0	0	0
Captive rhinoceroses										
Black		26	0	1	0	2	0	6	1	1
White		7	0	1	0	0	0	1	0	1
One-horned		1	0	0	0	0	0	1	0	1

[a] Free-ranging black rhinoceroses were captured in the drainages of the Kachowe and Chenje Rivers in the Chewore Safari Area, at Mana Pools National Park, on the Zambezi escarpment, from unspecified or scattered locations in Zimbabwe, or from Damaraland in Namibia.

[b] Number of rhinoceroses tested that had antibody titers ≥ 1:100 to *L. interrogans* serovars *autumnalis* (aut), *bratislava* (brat), *canicola* (cani), *grippotyphosa* (gripp), *hardjo* (hard), *icterohaemorrhagiae* (ict), *pomona* (pom), and *tarasovi* (tar).

rum samples were frozen within 48 hr of the time they were obtained, maintained frozen at or below −10°C, and shipped frozen or on ice to the National Animal Disease Center in Ames, Iowa.

Serologic examination

Serum samples were tested for antibodies against *L. interrogans* by microscopic agglutination testing for *autumnalis, bratislava, canicola, grippotyphosa, hardjo, icterohaemorrhagiae, pomona,* and *tarassovi* serovars.[5] An initial serum dilution of 1:12.5 and twofold dilutions were tested. Titers were recorded as the reciprocal of the highest dilution at which ≥50% of the leptospires were agglutinated. These dilutions were used for calculation of geometric mean titers (see Table 2).

Fluorescent antibody testing

Frozen liver tissue from four black rhinoceroses that died of acute hemolytic anemia and from four black rhinoceroses that died of unrelated diseases were tested using a fluorescent antibody test for the presence of *L. interrogans*. One- to 2-g samples were collected from frozen liver tissue and me-

chanically disrupted in 9 ml of phosphate-buffered saline. A further 1:20 dilution of the tissue suspension was prepared, and a 20-μl aliquot was placed on a glass slide, air dried, and fixed in acetone for 10 min. Tissue suspensions were stained with a combination of fluorescein-labeled rabbit anti-serovar *hardjo* and anti-serovar *bratislava* conjugate as previously described.[4] This combination conjugate stains most serovars of *L. interrogans*. Leptospires were identified by typical shape and specific fluorescence when examined by incident-light fluorescence microscopy.[10]

RESULTS

Thirty-eight of the 60 black rhinoceroses (63%) from Zimbabwe had titers of ≥1:100 to at least one serovar (Table 1). The prevalence of significant titers varied with the ecological conditions of the site of capture. Twenty-eight of 32 (88%) rhinoceroses captured in the relatively wet environment of the Zambezi River Valley had significant titers against one or more serovars of *L. interrogans*. In contrast, only two of 11 (18%) and none of three rhinoceroses captured in the savana–woodlands on the Zambezi es-

carpment and in arid areas of Namibia, respectively, had titers ≥1:100 to any of the eight leptospiral serovars tested. The prevalence of antibodies to individual serovars also varied among the capture sites. The most prevalent serovar in rhinoceroses captured along the Kachowe River (Chewore Safari Area) was *icterohaemorrhagiae*, whereas antibodies against serovar *tarassovi* were the most common in rhinoceroses captured in Mana Pools Park and along the Chenje River in the Chewore Safari Area (Fig. 1).

Overall, titers to *L. interrogans* in nonvaccinated captive rhinoceroses were low (Tables 1, 2). Six nonvaccinated rhinoceroses had titers ≥1:100 against serovar *icterohaemorrhagiae*. The highest titer of antibody against serovar *icterohaemorrhagiae* was 1:320 from a rhinoceros that died of acute hemolytic anemia.[11] Titers of ≥1:100 against serovar *icterohaemorrhagiae* occurred in three black rhinoceroses at the Denver Zoo, one of which had survived multiple mild episodes of hemolysis, one of which was exposed to rhinoceroses that had experienced hemolytic anemia, and one that was born to a vaccinated dam. A titer of 1:100 against serovar *tarassovi* was found in a recently imported black rhinoceros that was dying of liver failure of unknown etiology. Two rhinoceroses had antibody titers against serovar *grippotyphosa*, and one had a titer against serovar *bratislava*; none of these animals had clinical signs suggestive of leptospirosis.

Titers were available from four black rhinoceroses that died during hemolytic crises. These included the animal noted above (1:320 to serovar *icterohaemorrhagiae*), one rhinoceros with a titer of 1:25, also to serovar *icterohaemorrhagiae*, and two that lacked significant antibody titers against any of the eight serovars tested. Titers available from four rhinoceroses that had survived mild hemolytic anemia were also unremarkable; the highest was 1:100 to serovar *icterohaemorrhagiae* in a sample collected 7 yr after the hemolytic anemia. The highest titers in three black rhinoceroses exposed to rhinoceroses with hemolytic anemia were against serovar *icterohaemorrhagiae*, with a mean titer of 1:25 (the highest was 1:200).

Two of seven captive white rhinoceroses had titers ≥1:100: one against serovar *icterohaemorrhagiae* and one against serovar *bratislava*. The greater Asian one-horned rhinoceros had a titer of 1:200 to serovar *icterohaemorrhagiae*. Neither hemolytic anemia nor any other evidence of leptospiral infection was noted in these rhinoceroses nor has hemolytic anemia been noted in these species.

Because postvaccination serum samples were collected on an opportunistic basis from the black rhinoceroses, individual animals were not followed on a serial basis. Therefore, mean titers are presented in Table 2. Antibody titers to each of the serovars included in the vaccine increased after vaccination. Antibody titers also increased to serovars *autumnalis* and *bratislava*, which were not present in the vaccine, because of the cross-reactivity of anti-leptospiral antibodies. The titers against each serovar peaked between 14 days and 4 mo after vaccination.

Leptospires were detected by fluorescent antibody test in the livers of three of four rhinoceroses that died of hemolytic anemia and in an adult female rhinoceros that died with ulcerative skin disease. Leptospires were not detected in the liver of the other two rhinoceroses (one hemolytic and one nonhemolytic) tested.

DISCUSSION

Leptospirosis is a disease of worldwide distribution that is commonly associated with moist environments and frequently with rodent transmission. Leptospires, transmitted in rodent urine or urine of other infected animals, are most viable outside the host when shed into a neutral to slightly alkaline muddy or moist environment. The pans, ponds, and other water sources used by the black rhinoceroses of the Zambezi River Valley provide such an environment,

Table 2. Microscopic agglutination titers for leptospirosis in captive black rhinoceroses.

Status	No. of samples	Geometric mean titers[*]							
		aut	brat	cani	gripp	hard	ict	pom	tar
Nonvaccinated	30	0.1	0.8	0.5	0.4	0.04	2.2	0.2	0.1
Vaccinated[b]									
0.5–1.5 mo	3	5.3	6.6	6.6	7.0	4.3	7.7	6.6	0.0
4 mo	4	3.3	6.5	7.0	5.0	5.8	9.5	8.3	0.5
6 mo	4	1.5	3.3	4.0	4.0	3.8	6.3	3.8	0.5
6–12 mo	5	2.4	2.8	6.2	5.6	2.6	6.4	6.0	0.0
15 mo	2	0.5	3.5	2.5	3.5	2.0	4.0	3.5	1.0
24 mo	1	0.3	3.8	1.3	2.8	1.0	4.5	2.8	0.5
Rhinos surviving hemolysis[c]	4	0	1.0	0.2	0	0	1.5	0	0.3
Rhinos exposed to hemolytic rhinos[d]	4	0	0.8	0.8	0	0	2.0	0.2	0

[*] Titers expressed as dilutions: 1 = 1:12.5; 2 = 1:25; 3 = 1:50; 4 = 1:100; 5 = 1:200; 6 = 1:400; 7 = 1:800; 8 = 1:1,600; 9 = 1:3,200; 10 = 1:6,400.

[b] Time elapsed between vaccination and sampling.

[c] Average interval between last hemolytic event and sampling was 36 mo.

[d] Average interval between exposure to a rhinoceros experiencing hemolysis and sampling was 27 mo.

and this may explain, in part, the higher prevalence of antibodies against *L. interrogans* in rhinoceroses captured in those areas. Although exposure to leptospiral organisms appears to be common in the Zambezi River Valley, the dominant serovar varies from area to area. Serovar *icterohaemorrhagiae* was the predominant serovar in the Kachowe River drainage. Serovar *tarassovi* was predominant in the Mana Pools National Park further to the west and in the Chenje River drainage further to the east. The animals from along the Chenje River were captured closer to the base of the Zambezi escarpment and at slightly higher elevation than those along the Kachowe River or Mana Pools. Samples from rhinoceroses captured at the Zambezi escarpment sites indicate the black rhinoceroses from these savanna–woodland areas were generally less commonly exposed to leptospires. Although the influence of elevation, soil type, precipitation, and rodent fauna could not be distinguished in this study, serovars appear to be specific to particular drainages or river valleys. Samples from scattered locations or for which location was not recorded showed no obvious pattern of exposure.

There was no serological evidence of leptospiral exposure of black rhinoceroses captured in the Damaraland area of Namibia. The arid climate in Damaraland results in very low numbers of rodents, except following the rare heavy rain, and little or no vegetation near water sources. The scarcity of water sources (often only small seeps) and the poor quality of water (often rather saline) also may influence the survival of leptospiral organisms.

Complete epidemiological evaluations of these data are complicated by small sample size and the limited capture location data on some black rhinoceroses and because the extensive home ranges of rhinoceroses may overlap several ecologically distinct areas. However, this study does suggest that the prevalence of exposure to leptospiral serovars may be a local phenomenon. Thus, some wild animals may have natural exposure and limited resistance to infection with one or more serovars but may have no resistance to others.

Diagnosis of leptospirosis is often difficult. Serologic examination is often helpful, but antibodies may not be present in animals that die of acute leptospirosis. Other diagnostic methods to detect leptospiremia,

leptospiruria, and the presence of spirochetes in tissues require specialized techniques and are dependent on the number of organisms present. Leptospiral culture is difficult and requires specialized media. For diagnostic purposes, the fluorescent antibody test offers a practical and reasonably sensitive method for detecting leptospires in tissues and body fluids. It is a rapid method and can be performed on frozen tissues. However, fluorescent antibody conjugates are usually nonspecific and, therefore, the infecting serovar cannot be determined using this technique. Therefore, combinations of serology and fluorescent antibody testing are often used to diagnose leptospirosis.

Leptospires were detected, using the fluorescent antibody test, in the liver of three of four black rhinoceroses that died of hemolytic anemia. Two of these three rhinoceroses lacked detectable serum antibodies against the serovars of *L. interrogans* tested. The third rhinoceros had a titer of 1:320 to serovar *icterohaemorrhagiae* but was negative on other pre- and postmortem leptospiral evaluations.[12] The lack of anti-leptospiral antibodies in these rhinoceroses may be due to a peracute infection, resulting in death, before the rhinoceroses produced agglutinating antibody. Also, these rhinoceroses may have been infected with serovars other than those tested for in this study. However, these results emphasize the importance of saving frozen tissue from all black rhinoceros necropsies for leptospiral fluorescent antibody testing.

In nine of 31 cases of hemolytic anemia in captive black rhinoceroses there has been evidence of infection with *L. interrogans*.[3,7,9,11–13,15] Diagnosis was based on 1) elevated titers (1:8,000) to serovar *icterohaemorrhagiae* in one case[7] and to serovar *grippotyphosa* (1:6,400 and 1:12,800) in two others,[9] 2) spirochetemia in one rhinoceros,[15] 3) the presence of silver-stained spirochetes in tissues of one rhinoceros,[13] 4) and positive fluorescent antibody results of three black rhinoceroses as reported here. Not all cases of hemolytic anemia in black

rhinoceroses have been associated with leptospirosis. Therefore, studies are ongoing to identify other factors that may predispose animals to red blood cell instability and allow numerous factors (including leptospirosis) to preciptate a hemolytic crisis.[6,14]

Titers in nonvaccinated captive black rhinoceroses were generally unremarkable; the highest titers were those against serovar *grippotyphosa* in one animal and those against serovar *icterohaemorrhagiae*, which indicated frequent exposure. In the United States, rats and raccoons are reservoirs for serovars *icterohaemorrhagiae* and *grippotyphosa*, respectively,[1] and therefore, exposure of captive rhinoceroses to these serovars is probably unavoidable. Titers from black rhinoceroses that survived mild hemolytic anemia or were exposed to rhinoceroses that had hemolytic anemia were not markedly different from those of normal nonvaccinated black rhinoceroses. Possible explanations for this include 1) leptospirosis was not involved in these cases or 2) the prolonged period between the hemolytic anemia episode and opportunistic sampling (an average of 36 mo in the hemolytic animals and 27 mo for the exposed ones) may have resulted in waning antibody titers.

In black rhinoceroses vaccinated with a vaccine containing five leptospiral serovars, postvaccination titer responses were similar to those seen in domestic species. Interpretation of these results is complicated by the failure to sequentially follow titer responses in individual animals and by low numbers of samples in several postvaccination time periods. However, the titer increases were biologically significant and peaked within 4 mo of vaccination. At 15–24 mo postvaccination, titers had decreased to nearly baseline levels. We know of only two adverse reactions in vaccinated animals: a possible anaphylactic reaction in a rhinoceros that recovered following apparent weakness and sternal recumbancy immediately postvaccination and an injection site abscess in one rhinoceros at the St. Louis Zoo. On the basis of the hemolytic episodes in which leptospi-

rosis was suggested, the high fatality rate of the hemolytic syndrome (75%), and the lack of frequent or serious adverse reactions to vaccination with leptospiral vaccines. We recommend that all captive black rhinoceroses be vaccinated with a leptospiral vaccine containing at least five serovars. An initial series of two vaccinations at least 2 wk apart is recommended. Until further studies more clearly establish postvaccination humoral immune responses and events, vaccination should be performed at 6-mo intervals.

CONCLUSIONS

The serologic data in this report document a low level of exposure of captive black rhinoceroses in North America to leptospirosis and a relatively common natural exposure to area-specific serovars in the Zambezi Valley of Zimbabwe, with no apparent exposure in the Damaraland, Namibia. Microscopic agglutination titers in captive rhinoceroses are generally low and apparently variable in infected animals. Fluorescent antibody testing offers an improved method for the diagnosis of leptospirosis and indicates that infection with *L. interrogans* has occurred in several black rhinoceroses with hemolytic anemia. Further work is necessary to determine the role that red blood cell stability and leptospirosis may play in precipitating hemolysis in this species.

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