POTENTIAL FOR SERODIAGNOSIS OF TUBERCULOSIS IN BLACK RHINOCEROS (*DICEROS BICORNIS*)

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Abstract: A case of fatal *Mycobacterium tuberculosis* infection was diagnosed postmortem in a captive 33-yr-old male black rhinoceros (*Diceros bicornis*) after a nonspecific illness in April 2013. Retrospective testing of sera from this individual revealed that it had been seroreactive by ElephantTB STAT-PAK, dual-path platform VetTB, and multi-antigen print immunoassay for over 12 yr prior to death. Although samples collected at the time of intradermal tuberculin test performed in October 2000 were nonreactive in all three serologic assays, the animal appeared to seroconvert approximately 2.5 wk after the skin test administration. The antibody response remained detectable for the duration of the animal's life (12+ yr), indicating ongoing immunologic stimulation. The current case report supports the use of serologic assays for diagnosis of TB in black rhinoceros and may provide information for earlier detection. However, further research is needed to develop tools for recognition of mycobacterial infections in rhinoceros.
Key words: Black rhinoceros, *Diceros bicornis*, *Mycobacterium tuberculosis*, serological tests, tuberculosis.

BRIEF COMMUNICATION

Tuberculosis (TB) is a global concern for rhinoceros, especially in managed care. Infection and disease caused by *Mycobacterium tuberculosis* or *Mycobacterium bovis* have been reported sporadically in rhinoceros in the United States, Europe, India, and South Africa.[1–3,6,8,9,11,17,18] Since TB is an important regulatory and zoonotic, as well as interface, disease, the impact of this alien pathogen on conservation and management of endangered species in captivity and in free-ranging populations is a significant concern.[3,12,13] However, detection of mycobacterial infection in rhinoceros continues to present challenges because of the lack of validated antemortem diagnostic tools.[13]

The frequency of TB occurrence in rhinoceros is unknown since most animals are not tested routinely. This creates the potential for spreading disease during translocation or movement of animals between facilities or populations. In a survey of zoologic facilities, the majority (65%) of

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institutions reported not testing their captive rhinoceros.[4] TB testing of translocated rhinoceros is not required by most countries, even if animals originate from TB-endemic areas. For example, the rhinoceros population in Kruger National Park (a TB-endemic area) is estimated at 9,000 animals, of which up to 300 rhinoceros may be translocated in any given year (Buss, pers. comm.). Rhinoceros in zoos may also be at risk of disease transmission from other species with TB.[9] In cases where individuals are screened for TB, there is a lack of standardized tests, which complicates interpretation. Intradermal tuberculin testing has been reported to be unreliable in rhinoceros, while mycobacterial culture of nasal discharge, tracheobronchial or gastric lavage samples, feces, and urine may only yield positive results in some infected animals.[3,4,9,11,13]

Serologic assays appear to be useful for detection of TB in black rhinoceros.[2] These tests are rapid and only require a single serum sample; however, test sensitivity in rhinoceros species has not been determined. Interferon-γ release assays show promise as a screening tool for rhinoceros but require fresh whole blood samples, species-specific immunoreagents, and a laboratory setting.[14,15] With less than 5,000 black rhinoceros (*Diceros bicornis*) remaining globally and over 100 individuals managed in North American zoos, techniques to increase sensitivity of available methods for diagnosing mycobacterial infection in rhinoceros are needed. The present report describes a new TB case due to *M. tuberculosis* in a black rhinoceros and potential diagnostic value

Figure 1. Gross pulmonary lesions associated with *Mycobacterium tuberculosis* infection in black rhinoceros.

of serologic tests to detect disease in this host species.

A captive 33-yr-old male black rhinoceros was found dead in its enclosure on 03 April 2013 after a nonspecific illness. Beginning in January 2013, this animal had exhibited clinical signs of lethargy, and appetite and fecal production were both decreased. Initial blood results showed anemia (hematocrit 24%), leukocytosis (white blood cell count 15,200 cells/μl), and hypophosphatemia (1.3 mg/dl). The rhinoceros developed dermal ulcers over the hips and hocks in early February. Intermittent signs of decreased appetite, lethargy, loose fecal consistency, and dermal ulcers were reported through the end of March. The day prior to death, the animal's eyes were sunken and respiratory dyspnea was observed, along with difficulty rising to its feet. The animal died overnight and underwent complete necropsy at the zoo.

Gross pathologic findings included multifocal coalescing cream-colored pulmonary lesions with irregular borders (Fig. 1). Microscopically these areas were characterized by necrosis with calcified cellular debris mixed with dense infiltrates of lymphoplasmacytic cells, fibrocytes, and aggregates of epithelioid macrophages with some multinucleated giant cells. Many of the macrophages and multinucleated giant cells contained variable numbers of acid-fast–positive intracytoplasmic bacteria. Culture of lung lesions yielded *Mycobacterium tuberculosis*, confirmed by IS6110 polymerase chain reaction. The spoligotype 000000000003731 of this isolate is uncommon in animals but Beijing strain is found in humans worldwide (National Veterinary Services Laboratories, Ames, Iowa 50010, USA).

Upon confirmation of TB, an epidemiologic investigation was initiated using retrospective analyses of banked serum samples, medical records, and testing of contacts. Banked sera were analyzed initially as blind-coded samples along with those collected from three living black rhinoceros that were in contact with this case at the zoo using ElephantTB STAT-PAK, dual-path platform (DPP) VetTB, and multi-antigen print immunoassay (MAPIA) (Chembio Diagnostic Systems, Inc., Medford, New York 11763, USA). Serum samples taken from the infected animal 5 days and 2 mo prior to death showed strong antibody reactivity in all three assays, with ESAT-6 and CFP10 antigens being recognized (immobilized in MAPIA as single proteins and as polyepitope fusions, E6/P10 and F10), whereas no antibodies to these antigens of *M. tuberculosis* were detected in the three living animals (Fig. 2). To determine time of seroconversion, additional banked samples from the infected rhinoceros dating back to 2005 (collected in the current zoo) and 2001 (collected in the previous institution) were analyzed. As shown in Table 1, serum taken on 31 October 2000 at the time of intradermal injection of tuberculin-purified derivative (PPD) was nonreactive, whereas serum collected 2.5 wk later (17 November 2000) was strongly reactive in all antibody assays. Additional samples collected during 2001 and in 2005 were seroreactive.

In order to determine possible history of exposure, retrospective analyses of records were conducted. This black rhinoceros was born in a Canadian zoo in 1979 and was moved to several facilities in the United States between September 1980 and May 2001, when it was transferred to the final zoo. Prior to arrival, the animal had been housed at a large zoologic institution, where it had potential contact between June 1983 and March 1984 with a female black rhinoceros imported from Europe. The imported animal died in March 1984 and necropsy revealed the presence of lung lesions consistent with TB. However, culture results were unavailable from this case. There was no other indication of exposure to animals or facilities with a history of TB.
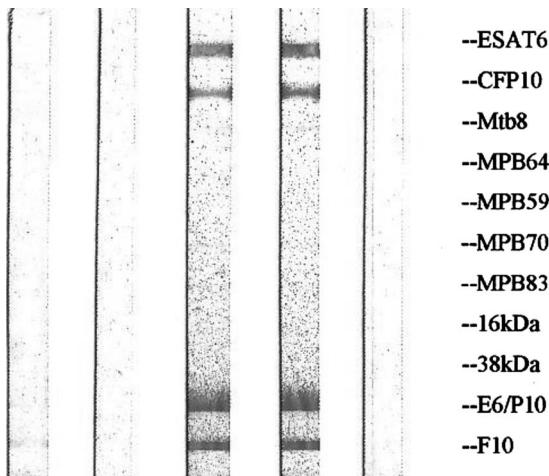
Antigens (right column):
--ESAT6
--CFP10
--Mtb8
--MPB64
--MPB59
--MPB70
--MPB83
--16kDa
--38kDa
--E6/P10
--F10

Figure 2. Multi-antigen print immunoassay results for *Mycobacterium tuberculosis*–infected black rhinoceros and contact rhinoceros. Antigens immobilized onto nitrocellulose membrane are shown to the right. Antibodies were detected using horseradish peroxidase–conjugated protein A as described previously.[2] Lanes 1, 2, and 5 show results obtained with sera from three contact rhinoceros collected in May 2013. Lanes 3 and 4 show results obtained with samples collected from the infected rhinoceros 2 mo and 5 days prior to death in April 2013, respectively.

Based on medical records from the previous institution, the rhinoceros that died in 2013 had negative intradermal tuberculin tests in April and July 1984. A subsequent intradermal tuberculin test in November 2000 was negative. A "suspect" reaction was reported to bovine PPD injected intradermally behind the ear on 07 April 2001. A comparative tuberculin test was performed in the caudal fold 3 days later with a 1-mm increase in skin thickness recorded to avian PPD and a 2-mm increase to bovine PPD. No further skin tests

were performed prior to transport in May 2001 to the current facility. No previous history of TB was reported at the zoo where the *M. tuberculosis*–infected rhinoceros died. However, in order to determine possibility of transmission between rhinoceros at this facility, screening for mycobacterial infection was performed on three living rhinoceros at the zoo, which were in contact with the infected individual before its death in 2013. All three animals were seronegative when tested in May 2013 and again in May 2014. Furthermore, bronchial lavage mycobacterial cultures collected by bronchoscopy in May 2014 from each of the three rhinoceros were negative. These animals remain clinically healthy at the time of this writing.

This report describes a fatal TB case due to *M. tuberculosis* in a captive black rhinoceros. Retrospective studies using three serologic assays, ElephantTB STAT-PAK, DPP VetTB, and MAPIA, and banked samples collected from the animal between 2000 and 2013 demonstrated the potential for early TB serodiagnosis in this host species. Serologic tests have previously shown promise for antemortem diagnosis of pathogenic mycobacteriosis in several zoo species, although only limited efforts have been made to evaluate accuracy and utility of these tests in rhinoceros.[2,5,16] In the present study, antibodies to *M. tuberculosis* complex–specific antigens were detectable in the infected black rhinoceros over 12 yr prior to death, further supporting the value of serology for improved TB screening programs in zoo settings.

Although the presumed exposure may have occurred as long as 30 yr prior to diagnosis of TB at necropsy, there was no indication of infection prior to a "suspect" result on intradermal tuberculin test performed in April 2001.

Table 1. Antemortem diagnostic test summary for *Mycobacterium tuberculosis*–infected black rhinoceros.

Date	Tuberculin skin test	STAT-PAK	DPP[a] VetTB	MAPIA
13 Apr 1984	Negative	ND	ND	ND
30 Jun 1984	Negative	ND	ND	ND
31 Oct 2000	Negative	Nonreactive	Nonreactive	Nonreactive
17 Nov 2000	ND	Reactive	Reactive	Reactive
18 Jan 2001	ND	Reactive	Reactive	Reactive
31 Jan 2001	ND	Reactive	Reactive	Reactive
04 Apr 2001	Suspect	Reactive	Reactive	Reactive
10 Apr 2001	Suspect	Reactive	Reactive	Reactive
17 Oct 2005	ND	Reactive	Reactive	Reactive
24 Jan 2013	ND	Reactive	Reactive	Reactive
28 Mar 2013	ND	Reactive	Reactive	Reactive

[a] DPP indicates dual-path platform; MAPIA, multi-antigen print immunoassay; ND, not done.

However, on retrospective serologic testing, there was evidence of seroconversion 5 mo before (November 2000). It is recognized that the rhinoceros could have had an unknown TB exposure through contact with another infected animal or human prior to 2000, especially since the *M. tuberculosis* strain isolated is commonly found in humans. While serum collected on the day of PPD administration was negative, the next sample taken 17 days later showed a strong antibody reaction. One possible explanation is that intradermal tuberculin administration may have stimulated a B-cell memory response, thus leading to seropositive results in the STAT-PAK, DPP, and MAPIA tests. Another reason for the apparent change is that the seronegative result prior to PPD administration may have been a false negative test, in which antibodies were below the level of assay detection. This could be due to degradation of antibodies due to long storage period or artifacts introduced by incorrect sample handling or identification. It cannot be ruled out that disease progression may have contributed to the apparent seroconversion detected following the PPD injection. Regardless, this serologic response was detectable for the duration of the animal's life (12+ yr). It is likely that this animal had been infected for years and the bacillary load reached a threshold that led to persistent immunologic response. Previous studies on elephant TB have also demonstrated that serologic tests can identify *M. tuberculosis*–infected animals many years prior to culture-based diagnosis.[5]

Boosting of humoral responses by intradermal tuberculin tests have been reported for other species. Cervids experimentally infected with *M. bovis* had significantly increased antibody responses detected by enzyme-linked immunosorbent assay, immunoblot, MAPIA, or CervidTB STAT-PAK after comparative cervical intradermal tuberculin tests.[7,19] Interestingly, some *M. bovis*–infected cattle had detectable responses to additional bands in MAPIA only after skin testing, thus increasing the proportion of antibody-positive animals and leading to enhanced sensitivity of the serologic assay.[10] Sporadic cases of TB in rhinoceros in managed care have been reported worldwide, with the majority diagnosed postmortem.[1–3,6,8,9,11,17,18] Anecdotal data suggest that rhinoceros may have been infected by humans or other animals in the same location. These findings support the need for increased surveillance and improved antemortem diagnostic testing for TB in rhinoceros. However, there is a lack of standardized methods for testing these species.[4,13]

Since rhinoceros may be infected for years prior to diagnosis, earlier detection of TB is required to identify infected animals to prevent transmission and inform management decisions. The presented findings suggest that serologic assays that detect antibodies to specific mycobacterial antigens have the potential to improve our ability to detect TB in rhinoceros. Future research should focus on identifying and standardizing specific methods for antemortem diagnosis of TB in these species.

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