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APPLICATION OF ELEPHANT TB STAT-PAK ASSAY AND MAPIA (MULTI-ANTIGEN PRINT IMMUNOASSAY) FOR DETECTION OF TUBERCULOSIS AND MONITORING OF TREATMENT IN BLACK RHINOCEROS (*DICEROS BICORNIS*)

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Abstract: Many wildlife species including rhinos are susceptible to infection with *Mycobacterium tuberculosis* or *M. bovis*. Antemortem diagnostic testing in large exotic hoof stock species has been limited by challenges associated with test administration, sample collection, and interpretation. Hence, a simple, rapid, blood-based test is needed. Two confirmed *M. tuberculosis*-infected black rhinoceros and one exposed suspect were evaluated for antibody responses using a lateral-flow rapid test (ElephantTB STAT-PAK) and multi-antigen print immunoassay (MAPIA). All three animals were seropositive by both tests. MAPIA detected antibodies to ESAT-6, CFP10, and MPB83 antigens. When the rhinos were treated with antitubercular therapeutics, their antibody responses gradually declined. One rhinoceros died approximately 9 mo after initiation of treatment and showed an increase in antibody titer shortly before death. The other two rhinoceros, which were treated for 1 and 2 yr, respectively, had no clinical signs or positive culture for *M. tuberculosis* at the time of necropsy performed 2 or 6 yr later for unrelated reasons. The antibody levels in these rhinos continued to be significantly decreased. The findings suggest that the ElephantTB STAT-PAK and MAPIA may be useful tools to detect *M. tuberculosis* infection and monitor treatment in black rhinoceros.

Key words: Black rhinoceros, *Diceros bicornis*, *Mycobacterium tuberculosis*, serology, tuberculosis.

INTRODUCTION

Tuberculosis continues to be a significant concern in both captive and free-ranging wildlife. Due to its importance as a zoonotic and regulatory disease, accurate diagnostic testing is crucial for detection and control. This is even more critical if treatment of captive endangered species is being considered. Tuberculosis in rhinoceros (*Ceratotherium simum*, *Diceros bicornis*) has been documented.[13] Testing of rhinos is especially challenging, as their large size increases the difficulty of diagnostic sample collection and risks associated with handling and anesthesia. A survey to determine testing methods in captive rhinoceros found that 65% of responding institutions did not test their animals for tuberculosis. Those institutions that did test performed intradermal testing and/or culture of nasal discharge, feces, urine, and gastric lavage samples.[2] While the domestic animal standard of intradermal skin testing has been used widely to screen nondomestic animals, this test has not been uniformly administered, validated or standardized in most exotic species, including the rhinoceros.[2,11] Intradermal skin testing has been performed in rhinos utilizing several different antigens in various injection sites.[2,9,11] This method requires multiple immobilizations, is difficult to interpret, and has poor diagnostic performance in this species.[13] Therefore, a rapid and more efficient test requiring only one handling event is needed for identification of rhinoceros infected with tuberculosis.

Serologic techniques have recently shown diagnostic potential for a variety of both captive and free-ranging wildlife hosts.[5–8,14–16] The studies suggested that antibody detection assays being developed for tuberculosis in different animals must be tailored to each species, to identify immunodominant antigens during infection and, hence, achieve optimal performance. This can be accomplished by the multi-antigen print immunoassay (MAPIA) employing a wide array of test antigens to characterize a species' antibody reactivity patterns.[4] From MAPIA findings, a point-of-care test can be designed using lateral-flow technology to provide a rapid and convenient animal-side diagnostic tool.[7] Recently the ElephantTB STAT-PAK assay (Chembio Diag-

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nostic Systems, Inc., Medford, New York 11763, USA) has been developed using this approach.[6] The present report describes the use of MAPIA and ElephantTB STAT-PAK assays to detect antibodies to *Mycobacterium tuberculosis* in infected black rhinoceros and to monitor serologic response during treatment.

CASE REPORT

Humoral immune responses from three black rhinoceros with confirmed or suspected tuberculosis were characterized retrospectively. Rhinoceros 1 resided at one facility, while Rhinoceroses 2 and 3 were from the same institution. All available serum samples from each animal (total of 25) were tested using the ElephantTB STAT-PAK and MAPIA, as described previously.[6] A single serum sample collected at the time of death from three black rhinoceros confirmed negative for tuberculosis at necropsy were used as controls. Two of the rhinos were from a zoo with no cases of tuberculosis, and one was an exposed contact rhino who lived with Rhinoceros 1 for 2 yr. MAPIA results from all three negative rhinos showed no antibody response to any of the tuberculosis antigens.

Rhinoceros 1 from facility A

A 24-yr-old female black rhino was housed at a facility with a history of *M. tuberculosis* infection in an Asian elephant (*Elephas maximus*) and two mountain goats (*Oreamnos americanus*).[12] In 1998, a nasal wash obtained from the rhino was polymerase chain reaction (PCR) and culture positive for *M. tuberculosis*. Intradermal testing using bovine purified protein derivative (PPD) was positive in 1998 but not in 1994. Treatment was initiated using pyrazidamide, isoniazid, and rifampin in September 1998 and completed 1 yr later. In August 2000, weight loss and nasal discharge were observed. Despite additional treatment and supportive care, the rhino was euthanized in January 2001. The final diagnosis was atypical mycobacterial pneumonia, with no *M. tuberculosis* present on culture. Figure 1 shows the MAPIA results from Rhinoceros 1. This rhino developed a strong serologic response at the time of diagnosis. Several antigens were found to be reactive including ESAT-6, CFP10, and the fusion protein CFP10/ESAT-6. The next serum sample collected 8 mo after initiation of therapy showed a sharp decrease in the antibody levels, followed by further slow decline until the time of death in 2001. Similar results were obtained for this rhino by the ElephantTB STAT-PAK assay when measuring the antibody responses with the optical reader (Fig. 2).

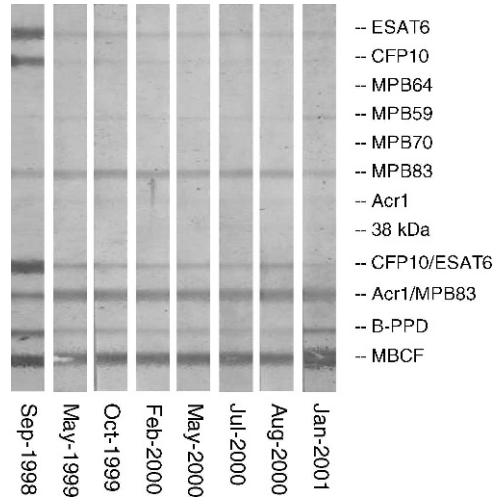

Figure 1. Multi-antigen print immunoassay (MAPIA) findings from Rhinoceros 1. Twelve specific mycobacterial proteins of *Mycobacterium tuberculosis* and *M. bovis* previously shown to generate antibody responses during tuberculosis are shown on the y-axis.[7] This rhino showed strong seroreactivity to antigens ESAT-6, CFP10, MPB83 and the fusion protein ESAT6/CFP10 at the time of isolating *M. tuberculosis* on culture. The bands found to correlate with active infection diminish markedly in response to treatment, with a continued slow decrease until the time of death in 2001.

Rhinoceros 2 from facility B

In January 1993, a 31-yr-old female black rhinoceros was diagnosed with *M. tuberculosis* infection based on positive cultures from sputum and gastric lavage. This individual had previously been treated prophylactically with isoniazid in 1981 for 1 yr after exposure to a tuberculin skin test–reactive exhibit mate. This female was also bred to this individual in 1985. Intradermal testing with bovine PPD was negative in 1989, but it reacted when tested in October 1992. Treatment was initiated with isoniazid and rifampin once culture results were received. Ethambutol and pyrazidamide were added to the treatment regimen and isoniazid discontinued based on results of culture sensitivities. Gastric lavage cultures collected in September 1993 and January 1994 were negative. Despite treatment, the rhino died in March 1994, and *M. tuberculosis* was cultured from a thoracic lymph node, tracheal exudates, and gastric lavage.[1] Rhino 2 was seronegative when originally tested in July of 1989 and strongly

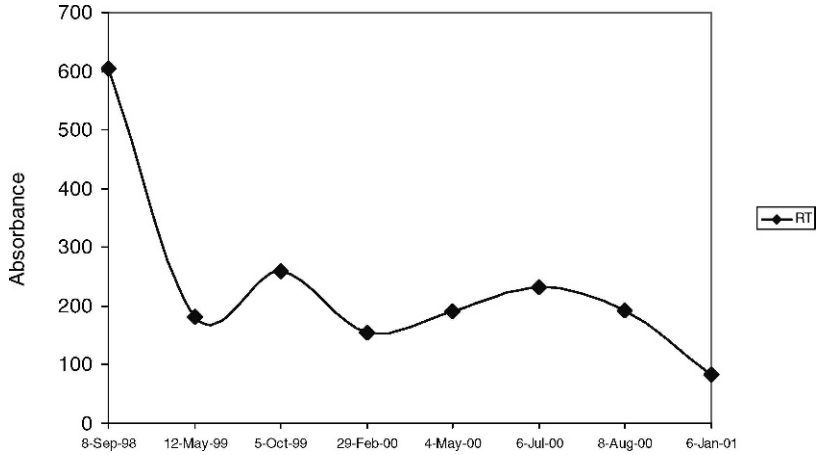

Figure 2. Results from the ElephantTB STAT-PAK for Rhinoceros 1. Results are consistent with those found using Multi-antigen print immunoassay (MAPIA) and shown in Figure 1.

seropositive in January 1993, when found to be culture-positive. The animal initially showed a decrease in antibody titer with treatment; however, the antibody response rebounded in the month of death. Figure 3 shows the reader-generated semiquantitative data obtained with the ElephantTB STAT-PAK assay for Rhinos 2 and 3.

Rhinoceros 3 from facility B

In January 1993, this 41-yr-old male black rhino was the only exhibit mate of Rhinoceros 2. This animal had a suspect intradermal reaction to bovine PPD in October 1992, although previous testing in 1988 had been negative. Because of the positive culture from Rhinoceros 2, it was started on prophylactic isoniazid in April 1993. A gastric lavage culture collected in September 1993 was negative. Rifampin was added to the treatment regimen in October after serum isoniazid levels were found to be below therapeutic in Rhinoceros 2. The total period of treatment was 26 mo. Between 1993 and 1998, Rhino 3 was anesthetized 10 times to collect bronchoalveolar and gastric lavage samples for mycobacterial cultures, Ziehl-Neelsen staining, and PCR. Mycobacterial cultures were performed by three different laboratories; none of the samples tested positive. This rhino was euthanized in 2001 due to severe osteoarthritis; no gross or histologic evidence of tuberculosis was found. Culture was performed on frozen lung tissue collected at necropsy and was negative for any mycobacterial growth. PCR was conducted on frozen lung tissue and was also negative. Figure 3 shows that Rhinoceros 3 became seropositive in June of 1994, 6 yr after being introduced to and 1.5 yr after the isolation of *M. tuberculosis* from the female. In MAPIA, the most reactive antigens in Rhino 3 were ESAT-6, CFP10, and MPB83 proteins, as well as CFP10/ESAT-6 fusion (Duncan, unpub. data, 2008). Similar to Rhino 1, a rapid decline in antibody titers after initiation of treatment was observed, and the antibody levels continued to decrease until its death in 2001 (Fig. 3).

Three negative control black rhinoceros tested by the ElephantTB STAT-PAK assay and MAPIA showed no presence of antibody in any of the serum samples.

DISCUSSION

This case report demonstrates potential application of the ElephantTB STAT-PAK and MAPIA assays for detecting tuberculosis and monitoring treatment in black rhinoceros. When developing a serologic assay for tuberculosis, it must be determined which antigens are most strongly associated with active disease in the species of interest. Since MAPIA utilizes multiple antigens of *M. tuberculosis* and *M. bovis*, this method is useful for determining serologic patterns of infected animals. In elephant studies, the most important antigen for detecting tuberculosis was ESAT-6, with CFP10 and MPB83 being also seroreactive.[6] The black rhinoceros included in this report provide an opportunity to follow antibody response to an array of antigens during active tuberculosis and treatment. ESAT6/CFP10 fusion showed the strongest serologic correlation with active disease. Antibodies to ESAT-6, CFP-10, and B-PPD antigens declined in response to therapy, while antibody levels for other proteins (MPB83, Arc1/MPB83, MBCF) did not change

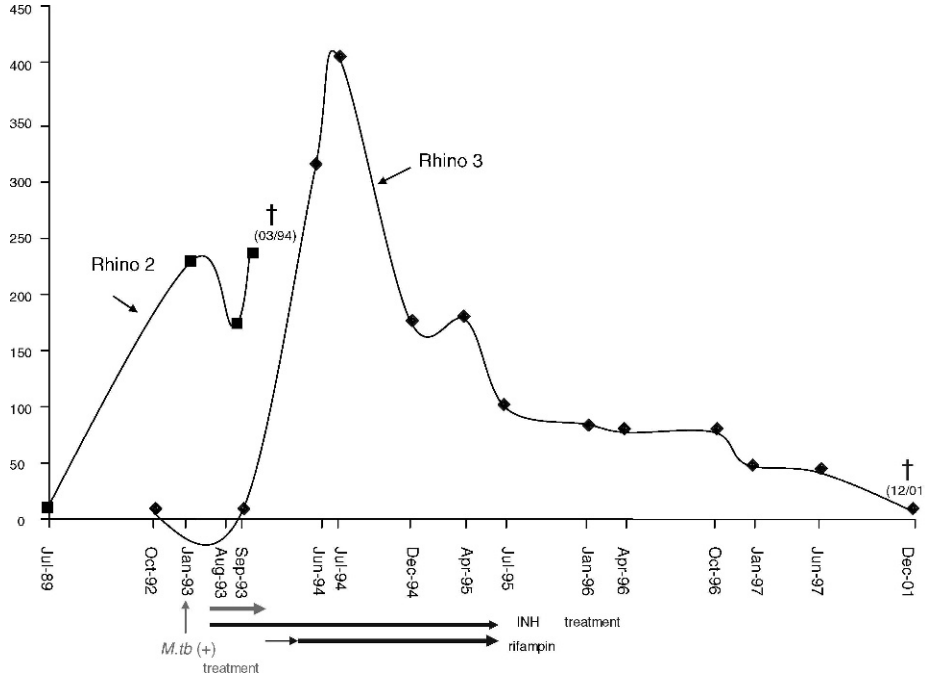

Figure 3. Semiquantitative ElephantTB STAT-PAK data obtained for Rhinos 2 and 3 by optical reader. Rhinoceros 2 was seronegative when originally tested in July of 1989 and strongly seropositive in January 1993, when found to be culture-positive. She initially showed a decrease in antibody titer with treatment; however, the absorbance level rebounded prior to death. Rhinoceros 3 was seropositive when tested in June of 1994. There is a rapid decline in antibody titers to the antigens ESAT6, CFP10, and MPB83 after initiation of treatment and a steady decline thereafter until his death in 2001.

significantly. Since the antigens ESAT-6, CFP10, and MPB83 are incorporated in the ElephantTB STAT-PAK assay, this version of the rapid test appears suited for use with rhinoceros.[6]

The ElephantTB STAT-PAK assay can detect IgG, IgM, and IgA antibodies within 20 min by visual evaluation of the presence or absence of a colored line on the test strip or semiquantitatively by measuring reflectance by a portable optical reader. Serum, plasma, and whole blood can all be utilized for testing; the sample volume needed is only 30 µl. As with MAPIA, a band of any density means antibody is present and the test is positive. The value for wildlife work is that the ElephantTB STAT-PAK kit can be performed while animals are still anesthetized, and additional diagnostic samples or other management decisions can be made. This test has been used to screen a wide variety of host species including elephants, deer, Eurasian badgers, brushtail possums, wild boar, nonhuman primates, and camelids.[5–8,14,16]

Results of this study show that these tests were able to document antibody levels at the time of positive culture confirmation of infection. Antibody titers declined in two successfully treated cases indicating that these assays may have prognostic value in monitoring therapy, similar to what has been shown in elephants.[6] Antibody levels to proteins ESAT-6 and CFP10 showed a gradual decrease in Rhinoceros 1 and 3, which eventually were found to be cleared of tuberculosis infection. Conversely, antibody levels in Rhinoceros 2 showed a sharp increase shortly before death, and tuberculosis infection was confirmed postmortem.

Over the last 2 decades, tuberculosis has caused significant morbidity and mortality among elephants, largely due to the lack of a screening test able to identify infected animals early enough that treatment can be effective and contact with other elephants avoided. In many zoologic facilities, large hoof stock species are housed in close proximity or cared for by the same staff, allowing opportunities for rhinoceros and other large hoof stock species to become exposed to the same diseases. Modern husbandry practices in many zoologic facilities mean more time spent training animals for medical and other behaviors, and potentially more time spent in the close contact necessary for transmission of certain

zoonotic diseases. Tuberculosis has been shown to be transmitted between elephants, rhinoceros, and other animals as well as humans in a zoologic setting.[3,10,12,13]

Early detection of infected animals is crucial to successful isolation and treatment. In infected elephants, tuberculosis could be identified by the ElephantTB STAT-PAK assay or MAPIA up to 42 months prior to detection by culture of trunk wash specimens, and long before the infected animals displayed any clinical signs.[6] In the present study, the culture-based diagnosis of tuberculosis in Rhinoceros 2 facilitated earlier suspicion of disease in exposed Rhinoceros 3, thus allowing for timely initiation of antitubercular therapy. Furthermore, MAPIA testing showed utility as a useful tool for monitoring the efficacy of treatment. This finding is especially critical for developing husbandry methods that will minimize spread of infection and standard protocols for antitubercular therapy in rhinoceros.

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