Highly Accurate Antibody Assays for Early and Rapid Detection of Tuberculosis in African and Asian Elephants

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Tuberculosis (TB) in elephants is a reemerging zoonotic disease caused primarily by Mycobacterium tuberculosis. Current methods for screening and diagnosis rely on trunk wash culture, which has serious limitations due to low test sensitivity, slow turnaround time, and variable sample quality. Innovative and more efficient diagnostic tools are urgently needed. We describe three novel serologic techniques, the ElephantTB Stat-Pak kit, multiantigen print immunoassay, and dual-path platform VetTB test, for rapid antibody detection in elephants. The study was performed with serum samples from 236 captive African and Asian elephants from 53 different locations in the United States and Europe. The elephants were divided into three groups based on disease status and history of exposure: (i) 26 animals with culture-confirmed TB due to M. tuberculosis or Mycobacterium bovis, (ii) 63 exposed elephants from known-infected herds that had never produced a culture-positive result from trunk wash samples, and (iii) 147 elephants without clinical symptoms suggestive of TB, with consistently negative trunk wash culture results, and with no history of potential exposure to TB in the past 5 years. Elephants with culture-confirmed TB and a proportion of exposed but trunk wash culture-negative elephants produced robust antibody responses to multiple antigens of M. tuberculosis, with seroconversions detectable years before TB-positive cultures were obtained from trunk wash specimens. ESAT-6 and CFP10 proteins were immunodominant antigens recognized by elephant antibodies during disease. The serologic assays demonstrated 100% sensitivity and 95 to 100% specificity. Rapid and accurate antibody tests to identify infected elephants will likely allow earlier and more efficient treatment, thus limiting transmission of infection to other susceptible animals and to humans.

Tuberculosis (TB) in captive elephants has been recognized as a reemerging zoonotic disease since at least the 1960s (22, 24, 26). In the past decade, growing numbers of elephant TB cases due to Mycobacterium tuberculosis or Mycobacterium bovis have been reported, presumably as a result of increased surveillance (11, 16, 25, 28). North American populations of African (Loxodonta africana) and Asian (Elephas maximus) elephants are declining, while captive breeding is historically poor. Efforts to maintain a self-sustaining captive population are hindered by TB-related issues. Many infected elephants may never exhibit clinical signs, whereas others with progressive disease may do so only in the terminal stages, thus making it difficult to recognize TB early (22, 24). Shedding of organisms during the preclinical period results in environmental contamination and presents a high risk of transmission to humans, elephants, and other mammals (11, 20, 27, 31).

The diagnostic value of the only existing antemortem testing method (i.e., culture of trunk wash samples) officially recommended by the United States Department of Agriculture (USDA) is limited by poor accuracy, slow turnaround time for sample processing, variable specimen quality, and sample acquisition logistics (11, 16, 25). Antibody detection assays have shown promising potential for identification of elephants infected with M. tuberculosis or M. bovis (10, 16). The new version of the Guidelines for the Control of Tuberculosis in Elephants 2008 (4) was recently approved by the United States Animal Health Association TB Committee. The document, including certain serologic tests, along with trunk wash culture for routine surveillance, is currently under USDA review to be adopted. This study describes the use of three novel serologic techniques (the ElephantTB Stat-Pak kit, multiantigen print immunoassay [MAPIA], and the dual-path platform [DPP] test) designed for early and accurate detection of TB in elephants.
well as BACTEC 12B vials, were inoculated with 0.5 ml of sample supplemented

MATERIALS AND METHODS

Animals. The study was performed with samples from 236 captive African and Asian elephants 2 to 68 years of age from over 53 different locations in the United States and Europe. The elephants were divided into three groups based on disease status and history of exposure (Table 1). The TB-infected group included 26 animals from 17 herds with culture-confirmed TB due to M. tuberculo-

TABLE 1. Study population

| Group                | Age range (yr) | No.          |
|----------------------|----------------|--------------|
| Culture-confirmed TB | 14-63          | African: 4   |
|                      |                | Asian: 22    |
|                      |                | Male: 4      |
|                      |                | Female: 22   |
| Exposed to TB        | 6-68           | 7            |
|                      |                | 56           |
|                      |                | 6            |
| Healthy or other     | 2-61           | 79           |
| disease*             |                | 68           |
|                      |                | 31           |
|                      |                | 116          |
|                      |                | 147          |

a Thirteen elephants with other diagnoses (chronic wasting syndrome, osteomyelitis, glomerulonephritis, chronic arthritis, or MOTT) were included.

saline (PBS) containing 0.05% Tween 20 (Sigma) and 2% (wt/vol) bovine serum albumin (PBS-TSA). After being blocked, the filters were placed into a 20-slot miniprotein II multiscience device (Bio-Rad), and individual sera (diluted 1:200 in PBS-TSA) were added to independent slots. After 2 h of incubation at 24°C with gentle rocking, the blots were washed three times with PBS and incubated with peroxidase-conjugated protein L (Sigma) diluted 1:2,500 in PBS-TSA for 1.5 h. The blots were again washed three times with PBS and developed for chemiluminescence in Supersignal detection reagent (Pierce Chemical Co).

MAPIA. The MAPIA test was performed as previously described (15) using a panel of 12 proteins of M. tuberculosis and peroxidase-conjugated protein G (Sigma), along with 3,3’,5,5’-tetramethyl benzidine (Kirkegaard & Perry Labora-

In conclusion, the study demonstrated the prevalence of M. tuberculosis and atypical mycobacteria in captive African and Asian elephants, with a majority of the cases being due to M. tuberculosis. The study results highlight the importance of surveillance and early intervention to prevent the spread of TB in these populations. Further studies are necessary to understand the epidemiology and pathogenesis of mycobacterial disease in Asian elephants and to develop effective control strategies.
RESULTS

Antibody responses in elephant TB. An initial indication that elephants can produce robust antibody responses to infection with *M. tuberculosis* was obtained by immunoblotting of sera collected over the course of >9 years from an elephant diagnosed with TB (Fig. 1). Antibody responses to a crude mycobacterial antigen preparation (i.e., *M. bovis* WCS) were detected 4 years prior to the isolation of *M. tuberculosis* from trunk wash samples. Notable in these responses were (i) a complex and progressive pattern of reactivity, (ii) consistency in responses to antigens of a distinct mass (e.g., ~24 kDa, ~32 kDa, and ~52 kDa), and (iii) a rapid decrease in reactivity to multiple antigens after initiation of antimycobacterial chemotherapy. To further analyze these findings, patterns of reactivity to a panel of recombinant proteins were evaluated by MAPIA (Table 2 and Fig. 2) using samples from the TB-infected and noninfected groups. All 26 of the infected elephants produced detectable serum immunoglobulin G (IgG) against one or more *M. tuberculosis* proteins, displaying variable profiles of antibody reactivity (Fig. 2). Among single proteins, ESAT-6 and CFP10 were the most frequently recognized molecules (92% and 81%, respectively), followed by MPB83 (58%) and others (4 to 19%) (Table 2). CFP10/ESAT-6 fusion protein was reactive with sera from all 26 infected elephants. Samples from all 147 noninfected elephants did not react with ESAT-6 or CFP10 alone or with the fusion protein. Sera from three of the four elephants with MOTT reacted with MPB83 antigen in the DPP VetTB test; these sera did not react with ESAT-6 or CFP10 antigens. Thus, ESAT-6 and CFP10 proteins appeared to be predominant and specific serological targets; use of a cocktail or fusion of the two proteins may provide an accurate antibody test for elephant TB.

Diagnostic performance of serologic assays. The diagnostic potentials of the ElephantTB Stat-Pak and MAPIA were initially demonstrated in a proof-of-concept study with a small number of samples (16). Recently, we have developed a novel point-of-care test based on the innovative DPP technology (Fig. 3). Reader device-generated data demonstrated clear-cut discrimination between the TB-infected and noninfected elephants using the DPP VetTB assay (Fig. 4). Good agreement was observed between test results obtained with the ElephantTB Stat-Pak, MAPIA, and DPP VetTB test. All 26 elephants in the TB group were CFP10/ESAT-6 antibody positive, thus yielding a sensitivity of 100% (95% CI, 84.0 to 100%) for each serologic test. None of the sera from noninfected elephants (*n* = 147) reacted with CFP10/ESAT-6 in MAPIA or the DPP VetTB assay, demonstrating a specificity of 100% (95% CI, 96.8 to 100%) for each test. As demonstrated with MAPIA, sera from three elephants with MOTT were reactive only with MPB83 antigen in the DPP VetTB test; therefore, use of a cocktail of antigens may provide an accurate antibody test for elephant TB.
however, this antibody reactivity was clearly distinguishable from the response to CFP10/ESAT-6 using the two-line format of the DPP VetTB (Fig. 3). The ElephantTB Stat-Pak kit showed seven false-positive results in the control group, resulting in a specificity of 95.2% (95% CI, 90.1 to 97.9%). Three of the seven reactors in the ElephantTB Stat-Pak assay were MOTT cases (presumably due to cross-reactivity with MPB83, as demonstrated with MAPIA and DPP VetTB), whereas three of the remaining four false-positive results were obtained with sera from elephants with arthritis. The diagnostic significance of the latter observation remains to be confirmed in future studies with larger sample numbers.

The specificities of the assays were additionally assessed with sera from four clinically healthy Asian elephants from which various species of atypical mycobacteria had been isolated during routine trunk wash culture testing. Sampling was performed every 3 to 6 months for each elephant over a period of 3 years. Trunk wash and blood specimens were obtained during the same week. Overall, 19 trunk wash samples collected from the four animals on different occasions were positive for

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**FIG. 3.** Antibody detection by DPP VetTB assay. The images represent examples of typical results obtained for elephant TB (A and B), MOTT due to *M. szulgai* (C), or noninfected control (D). Reflectance values in RLU generated by a DPP reader device for the MPB83 test band (gray bars) and the CFP10/ESAT-6 test band (black bars) are shown for each result. The proposed DPP VetTB test interpretation algorithm is shown on the right.

**FIG. 4.** Quantitative detection of serum IgG antibodies by DPP VetTB in culture-confirmed elephants. Reflectance values in RLU generated by a DPP reader with a CFP10/ESAT-6 test band are shown for sera from the 26 elephants with TB (solid circles) and for 100 randomly selected noninfected controls (open circles). A cutoff value of 3.0 RLU (dotted line) was established with the control sera as the mean plus 5 standard deviations.
Two to seven different mycobacterial species were collected from each elephant, including *Mycobacterium avium* complex (n = 5), *M. intracellulare* (n = 2), *M. gordoniae* (n = 2), *M. fortuitum* (n = 2), *M. abscessus*, *M. asiaticum*, *M. chelonae*, *M. flavescens*, *M. mucogenicum*, *M. nonchromogenicum*, *M. simiae*, and *M. terrae*. The matching 19 serum samples collected from the four elephants were tested by ElephantTB Stat-Pak, DPP VetTB, and MAPIA. No positive result was obtained with any of the sera, demonstrating that the presence of atypical mycobacteria in trunk wash specimens does not interfere with interpretation of the serologic assays for TB.

**Serology versus culture.** Banked pre-TB diagnosis sera were available for 16 infected elephants, which had been tested at least annually by trunk wash culture, providing the opportunity to retrospectively determine seroconversion times relative to antemortem culture results. Nine of these elephants were first diagnosed with TB by positive trunk wash culture. The other seven infected elephants were always trunk wash culture negative yet were confirmed TB infected by culture at a postmortem examination. In all 16 elephants, antibody responses were detected by the three serologic assays prior to culture-based diagnoses. Figure 5 demonstrates the times (range, 0.75 to 10 years; median, ~3 years) established between individual seroconversions detected by MAPIA and isolations of *M. tuberculosis* or *M. bovis* either from trunk wash samples or from tissue specimens at necropsy. The remaining 10 elephants in the TB-infected group, for which no prediagnosis sera were available for retrospective analysis, tested strongly seropositive at the time the first positive culture was obtained.

**Specific antibody responses in exposed, potentially infected, but trunk wash culture-negative elephants.** Of the 63 elephants in the TB-exposed group, for which no prediagnosis sera were available for retrospective analysis, tested strongly seropositive at the time the first positive culture was obtained.

**TABLE 3. Estimates of TB prevalence in captive elephant populations based on culture and serology data**

| Elephant species | Prevalence [% (95% CI)] | ESAT-6/CFP10 antibody [% (95% CI)] |
|------------------|--------------------------|-----------------------------------|
| African          | 4.4 (1.4–11.5)           | 4.4 (1.4–11.6)                    |
| Asian            | 15.1 (9.9–22.1)          | 21.9 (15.7–29.7)                  |
| Total            | 11.0 (7.5–15.9)          | 15.3 (11.0–20.6)                  |

* Trunk wash and/or postmortem testing.
* MAPIA and DPP VetTB data.
between TB prevalence estimates based on culture and serology (Table 3). Surprisingly, none of the four African elephants diagnosed with TB had ever produced a positive culture from multiple trunk wash specimens, whereas 15/22 (68%) infected Asian elephants were trunk wash culture positive. Therefore, if only antemortem culture testing was taken into consideration, the prevalence of disease would be greatly underestimated, being only 6.4% in total (0% in African elephants and 10.3% in Asian elephants).

Use of DBS specimens. The use of DBS specimens may enable collection of samples in remote locales, thereby facilitating broader surveillance, including in low-resource settings. We performed a DBS feasibility study with serum, plasma, and fresh whole blood collected from two *M. tuberculosis*-positive and two noninfected control elephants. Cellulose-based filter kits, BloodStain cards (Whatman, Inc.), originally designed for human diagnostic applications (7), were used in this pilot experiment. By comparing the DBS-eluted samples to the standard negative and positive control sera, it was determined that elephant TB-specific antibodies remained stable in DBS for at least 5 weeks (Fig. 6). This was demonstrated by ElephantTB Stat-Pak, DPP VetTB, and MAPIA. Procedures to produce DBS did not cause nonspecific reactions that could potentially result in false-positive results. There was no difference between serum, plasma, and whole blood with respect to both quantitative and qualitative characteristics of antibody detection (Fig. 6).

DISCUSSION

Elephant TB is a reemerging zoonotic disease caused primarily by *M. tuberculosis* (11, 22, 26). The *Guidelines for the Control of Tuberculosis in Elephants* define general principles and protocols for testing and treatment (33). Current diagnosis relies predominantly on trunk wash cultures, which is increasingly recognized to have serious limitations (11, 16, 25). This method, requiring three trunk wash samples collected on different days within 1 week, is labor-intensive, time-consuming, and expensive. Both the elephant and the caretaker or veterinarian must be well trained to obtain a trunk wash sample of acceptable quality. After sample collection, mycobacterial isolation and identification take 8 to 12 weeks, delaying notification of results. Increased numbers of trunk wash specimens for culture testing are required following a positive serologic assay for TB, as now recommended by the new *Guidelines for the Control of Tuberculosis in Elephants 2008* (4). In the present study, only 58% of the elephants with necropsy-confirmed TB had positive antemortem cultures from trunk wash specimens. This finding does not imply that the remaining 42% of the elephants never shed *M. tuberculosis*, as excreting of the organism by infected animals is inherently intermittent (3, 25). Also, human studies have demonstrated that patients with sputum smear-negative pulmonary TB, commonly considered low-risk sources of infection, are capable of transmitting the disease (1, 32).

Most elephants with active TB display no clinical signs, making it even more difficult to suspect disease (22, 25). In fact, only 15% of the culture-positive cases reported in this study had TB-suggestive symptoms prior to the time of diagnosis. Therefore, rapid and more efficient detection of infected animals is crucial to improve control of TB in elephants and other zoo species, as early diagnosis allows timely initiation of chemotherapy and quarantine to prevent transmission. Using sera serially collected over multiple years from 236 captive elephants, we evaluated the diagnostic potentials of three serologic techniques designed for early and accurate identification of elephants infected with *M. tuberculosis* or *M. bovis*. The ElephantTB Stat-Pak, MAPIA, and the DPP VetTB test correctly identified all infected animals and produced no false-negative reactions, thus demonstrating a perfect negative predictive value and 100% sensitivity. Importantly, the new serologic assays appeared to provide antemortem testing tools superior to the existing methods. Many infected elephants showed specific seroconversion years before shedding was detectable by culture of trunk wash samples.
were 100% for MAPIA and the DPP VetTB test and had never been trunk wash culture positive and had no wash samples. Moreover, the serologic assays identified a number of elephants with TB (confirmed postmortem) that had never been trunk wash culture positive and had no clinical signs of disease. The diagnostic test specificities were 100% for MAPIA and the DPP VetTB test and ~95% for the ElephantTB Stat-Pak assay.

Importantly, the results obtained with samples collected from culture-negative elephants that had been in contact with known TB cases indicate that serology may be a useful approach for more efficient surveillance of animals at risk for developing disease. Over the course of the present study, three TB-exposed elephants in different locations tested positive by ElephantTB Stat-Pak and MAPIA (with ESAT-6 antigen), although their trunk wash specimens were repeatedly culture negative. After one of these elephants died and other two were euthanized as strong suspects, \( M. \) \( \text{tuberculosis} \) strains (identical to those obtained from the source of infection in each case) were isolated from lung lesions collected at necropsy. These observations demonstrate the predictive value of highly sensitive antemortem tests for early diagnosis in “at-risk” groups. Thus, serology may be used to facilitate change in management practices in order to minimize infection risks to other animals, exhibition personnel, and the public (11, 27, 31).

Overall, the diagnostic performance of the DPP VetTB assay was equal to that of MAPIA and superior (in specificity) to the ElephantTB Stat-Pak kit. With the antigens studied, specific elephant TB serodiagnosis was closely associated with the presence of antibodies to the ESAT-6 and CFP10 proteins of \( M. \) \( \text{tuberculosis} \). The predominant serologic recognition of ESAT-6 has been reported for nonhuman primates infected with \( M. \) \( \text{tuberculosis} \) or \( M. \) \( \text{bovis} \) (2, 18), but not for other host species (5, 6, 12, 13, 14, 17, 19, 34–36). In contrast, several ElephantTB Stat-Pak false-positive results found in MOTT cases were due to the cross-reactive antibody responses against MPB83 protein, but not ESAT-6 or CFP10. Previous studies also demonstrated MPB83 seroreactivity in elephants infected with \( M. \) \( \text{szulgai} \) (9) or in cattle experimentally inoculated with \( \text{Mycobacterium kansasii} \) (37). Having this protein as a separate band in MAPIA or the DPP VetTB test appears to allow serological differentiation between elephant TB (antibodies against ESAT-6 and/or CFP10, alone or among others) and MOTT (anti-MPB83 antibody only) infections. Therefore, similar to MAPIA (16), the DPP VetTB assay can also be used under field conditions, if needed, as a faster and more convenient animal-side confirmatory tool for elephant TB.

The serologic assays performed well with blood specimens recovered from DBS, suggesting a useful sampling alternative for peripheral areas (e.g., field applications in Africa, Southeast Asia, etc.), where short-term storage or transportation of blood samples to a remote testing laboratory may be needed. This approach has been successfully utilized for serological surveillance of human viral infections in resource-limited countries (7, 29). For elephant-testing applications, a more extensive DBS validation with greater numbers of well-characterized samples will be required.

The high accuracy of elephant TB serodiagnosis was rather unexpected. Using similar immunoassays, we and others have reported much lower rates of TB detection in other species (5, 12, 17, 18, 34–36). The antibody test sensitivities ranged from 45% in brushtail possums, 49 to 51% in Eurasian badgers, or 73 to 75% in cervids to 77% in wild boar (3, 19), but they were never as high as the 100% found for the 26 infected elephants in the present study. This striking feature may stem from the complex biology of host-pathogen interactions, with variability in the immune responses between species. While the idea is only speculative, elephants normally have levels of peripheral blood monocytes significantly higher than those of ungulates, often in the range of 25 to 42% of circulating leukocytes (21, 23, 30), which may impact their immunity. The specific mechanisms for unusually robust antibody responses to TB in elephants remain unclear.

Despite their higher diagnostic potential, antibody assays are unlikely to replace culture methods in elephant testing. Isolation of mycobacteria from infected animals will always be useful to confirm the diagnosis, identify the strain (especially useful for molecular epidemiology studies), and generate drug susceptibility data (22). However, the management and control of TB in captive elephants and other nondomestic species will greatly benefit from early and rapid serodiagnosis. The cost of delayed diagnosis may be extremely high (16). Furthermore, undetected elephant TB may pose a serious zoonotic threat, with infection spillover from captive animals to free-ranging wildlife. This possibility is supported by findings of identical \( M. \) \( \text{tuberculosis} \) strains isolated from an infected elephant and an Addra gazelle housed in one facility (our unpublished observations) or from a group of elephants with TB and other species in the same zoo, including gibbon, tapir, and giraffe (11). Thus, timely recognition of disease followed by immediate and adequate interventions will likely prevent the spread of infection.

In conclusion, many African and Asian elephants with culture-confirmed TB produce robust antibody responses years before \( M. \) \( \text{tuberculosis} \) or \( M. \) \( \text{bovis} \) can be isolated from trunk wash samples. The serologic assays described in the present study have high diagnostic value for earlier detection of disease. The rapid and accurate identification of infected elephants will likely improve zoo TB control programs and allow more efficient treatment, thus limiting the transmission of infection to other susceptible animals and to humans.

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