

EVALUATION OF A MULTIPLE-ANTIGEN ENZYME-LINKED IMMUNOSORBENT ASSAY FOR DETECTION OF *MYCOBACTERIUM TUBERCULOSIS* INFECTION IN CAPTIVE ELEPHANTS

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Abstract: *Mycobacterium tuberculosis* has become an important agent of disease in the captive elephant population of the United States, although current detection methods appear to be inadequate for effective disease management. This investigation sought to validate a multiple-antigen enzyme-linked immunosorbent assay (ELISA) for screening of *M. tuberculosis* infection in captive elephants and to document the elephant's serologic response over time using a cross-sectional observational study design. Serum samples were collected from 51 Asian elephants (*Elephas maximus*) and 26 African elephants (*Loxodonta africana*) from 16 zoos and circuses throughout the United States. Infection status of each animal was determined by mycobacterial culture of trunk washes. Reactivity of each serum sample against six antigens was determined, and the linear combination of antigens that accurately predicted the infection status of the greatest number of animals was determined by discriminant analysis. The resulting classification functions were used to calculate the percentage of animals that were correctly classified (i.e., specificity and sensitivity). Of the 77 elephants sampled, 47 fit the criteria for inclusion in discriminant analysis. Of these, seven Asian elephants were considered infected; 25 Asian elephants and 15 African elephants were considered noninfected. The remaining elephants had been exposed to one or more infected animals. The specificity and sensitivity of the multiple-antigen ELISA were both 100% (91.9–100% and 54.4–100%, respectively) with 95% confidence intervals. *Mycobacterium bovis* culture filtrate showed the highest individual antigen specificity (95%; 83.0–100%) and sensitivity (100%; 54.4–100%). Serum samples from 34 elephants were analyzed over time by the response to the culture filtrate antigen; four of these elephants were culture positive and had been used to calculate the discriminant function. Limitations such as sample size, compromised ability to ascertain each animal's true infection status, and absence of known-infected African elephants suggest that much additional research needs to be conducted regarding the use of this ELISA. However, the results indicate that this multiple-antigen ELISA would be a valuable screening test for detecting *M. tuberculosis* infection in captive herds.

Key words: Elephant, *Mycobacterium tuberculosis*, ELISA, *Elephas maximus*, epidemiology, *Loxodonta africana*.

INTRODUCTION

Mycobacterium tuberculosis, the etiologic agent of human tuberculosis, has caused morbidity and mortality in elephants and is a potential zoonosis.^{3,23} Several recent infections in captive elephants in the United States have been reported.^{2,3,8,14,26,29} Most reports document infection in Asian elephants (*Elephas maximus*), although one infection in a captive

African elephant (*Loxodonta africana*) in France has been documented.³³ Elephant mycobacteriosis has become a disease of great concern to the public, zoo and circus communities, and regulatory authorities.³

In humans and domestic animals, screening for tuberculosis has been widely performed using the intradermal (i.d.) tuberculin test, the official test for live animals. Although this procedure has been described for nondomestic animals, systematic studies have not been conducted to evaluate biologic activity, optimal dose, or suitable injection sites in most species.²⁴ Other indirect laboratory tests that have been used for diagnosis of tuberculosis include lymphocyte transformation, gamma interferon, enzyme-linked immunosorbent assay (ELISA), and the blood tuberculosis test (BTB).^{11,13,17,19} These tests have not been validated, uniformly administered, nor consistently interpreted in most nondomestic species.²⁴ In elephants, i.d. and serologic test results have not correlated well with culture status.²⁵

Postslaughter testing remains the primary means of surveillance for cattle; however, this method is

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impractical and undesirable in endangered and threatened species such as the Asian elephant and African elephant. Recently, guidelines have been developed for tuberculosis control and diagnostic investigation in captive elephants³² that include mycobacterial culturing of respiratory secretions that are obtained from trunk wash material. Investigation of alternate testing modalities is being encouraged, however, in order to collect further information and to develop better tests.³² Mycobacterial culture, although highly specific, has limited sensitivity. Culture results may be falsely negative because of insufficient mycobacterial numbers in trunk secretions (<100 organisms/ml), inadequate collection procedures, contamination with other mycobacteria, intermittent mycobacterial shedding, and/or improper sample handling.²² Furthermore, mycobacteria grow slowly in culture media and require incubation for several weeks.³⁰ This delay between sample collection and diagnosis provides considerable opportunity for an infected animal to disseminate mycobacterial organisms, and it makes mycobacterial culture an impractical screening test for traveling elephants. Thus, a rapid, reliable, in vitro assay will be of considerable value in identifying and controlling tuberculosis in elephants.

At Colorado State University, an ELISA was developed for detection of humoral responses to *Mycobacterium bovis* infection.³⁴ This ELISA was developed using a mixture of protein A and protein G as conjugate for antibody binding because these proteins are not species specific.¹¹ The investigation presented here evaluated the validity of this multiple-antigen ELISA for screening of elephants for *M. tuberculosis* and documented the serologic responses of elephants over time.

MATERIALS AND METHODS

The ELISA procedure

The ELISA was patterned after previously reported assays,^{11,34} with a panel of antigens, instead of a single antigen, in order to improve sensitivity and specificity. This panel included a sterile, heat-killed culture filtrate (CF) of *M. bovis*, strain AN5 (National Veterinary Services Laboratory [NVSL], Ames, Iowa 50010, USA); purified protein derivative (PPD) from the standard United States Department of Agriculture bovine PPD tuberculin AN5 (NVSL); modified protein 70 (MPB) purified from the AN5 strain of *M. bovis* (P. R. Wood, CSIRO, Division of Animal Health, Animal Health Research Laboratory, Parkville, Victoria 3220, Australia); two lipoarabinomannan antigens from the virulent Erdman (ERD) and virulent H37 Ra (RA)

strains of *M. tuberculosis* (Tuberculosis Research Materials and Vaccine Testing Program, Colorado State University, Microbiology Department, Fort Collins, Colorado 80523, USA); and purified protein derivative (AVPPD) from *Mycobacterium avium* (NVSL).

Antigens were diluted with carbonate coating buffer (1.59 g of sodium carbonate and 2.93 g of sodium bicarbonate/L, pH 9.6). Antigen concentrations, on the basis of previous testing for *M. bovis* in cattle and cervids,^{11,34} were CF, 1.25 mg/ml; PPD, 10 mg/ml; MPB, 1 mg/ml; ERD, 0.5 mg/ml; RA, 0.5 mg/ml; and AVPPD, 10 mg/ml. Carbonate coating (50 μ l) was added to the first two wells, and each antigen (50 μ l) was added to subsequent wells on 12-column, eight-row, U-bottomed polystyrene microtitration plates (Dynex Technologies, Chantilly, Virginia 20151, USA). Plates were incubated overnight at 37°C, washed twice with phosphate-buffered saline solution (PBS; 0.9% NaCl, pH 7.2–7.4), washed once with deionized water, and blocked with 150 μ l/well superbloc reagent (Superblock, Pierce, Rockford, Illinois 61105, USA) for 5 min at 37°C. After blocking, wells were emptied and plates were allowed to dry for 3 hr.

Serum samples were diluted 1:100 with PBST (1% PBS containing 0.05% Tween 80 and 1% fetal bovine serum, premium cell culture grade, Bio-Whittaker, Walkersville, Maryland 21793, USA) on the basis of previous testing for *M. bovis*.^{11,34} For each test sample, 50 μ l of the sample was added to each of the wells in one row of the plate. Two positive control samples (*M. bovis*-infected bovine serum) and one negative control sample (fetal bovine serum), diluted 1:100 and 1:400 with PBST, were included on each plate. After all samples were added, plates were incubated for 1 hr at 37°C and then washed twice in PBS and twice in deionized water.

Protein A and G horseradish peroxidase conjugates (Protein A-HRP and Protein G-HRP, Prozyme, Inc., San Leandro, California 94577, USA) were diluted together in PBST to a concentration of 0.286 μ g/ml for each. The A and G conjugate mixture (50 μ l) was then added to each well. Plates were incubated for 1 hr at 37°C and then washed as above.

Frozen tetramethyl benzidine substrate (10 mg/ml in dimethyl sulfoxide) was thawed and diluted 1:100 with 0.1 M sodium acetate adjusted to a pH of 6 with 0.1 M citric acid buffer. Thirty percent hydrogen peroxide was added to achieve a final concentration of 0.0045%. The final solution (50 μ l) was then added to each well. Plates were covered and allowed to react at room temperature (approximately 25°C) for 12 min. The reaction was

stopped by adding 50 μ l of 2 M hydrogen sulfate to each well. Optical density (OD) was read (Microplate Manager Software, Bio-Rad, Hercules, California 94547, USA) at a wavelength of 450 nm.

For each test and control sample, the mean of the OD values for blank columns 1 and 2 (i.e., columns to which antigen had not been added) was subtracted from the mean of the OD values for the duplicate measurements for each antigen. This average value was then taken as a ratio versus the 1:100 positive control and versus the 1:400 positive control. These ratio values were recorded for each of the six antigens and are hereafter referred to as the OD values for each antigen.

Elephants

A request was made to elephant-owning institutions in the United States for both Asian elephant and African elephant serum samples as previous diagnostic tests have been evaluated without regard to species.^{3,25} Infections were definitively diagnosed by culture of *M. tuberculosis* from a trunk wash, processed at either the NVSL; the Orange County Public Health Laboratory, Santa Ana, California 92706, USA; the San Bernadino County Laboratory, San Bernadino, California 92412, USA; the San Francisco City and County Public Health Laboratory, San Francisco, California 94110, USA; the Arkansas Department of Health, Division of Public Health Laboratories, Little Rock, Arkansas 72205, USA; or Iowa State University, Ames, Iowa 50011, USA.

Animals designated as highly unlikely to be infected had trunk washes within 4 mo of serum sampling that were negative for mycobacterial culture of *M. tuberculosis*, no contact with elephants or other animals that had been diagnosed with *M. tuberculosis* within the last 5 yr, and no travel outside of the institution in the previous 5 yr. In order to avoid false-positive results, samples were excluded from analysis if i.d. tuberculin testing had been conducted within 6 mo of initial sampling, and in order to avoid false-negative results, samples were excluded if initial serum samples were collected after initiation of antituberculosis treatment.

The ELISA was conducted on samples from 77 elephants, including 51 Asian elephants and 26 African elephants, from 16 herds. Of these, seven Asian elephants had been diagnosed with *M. tuberculosis* infection via trunk wash, and one of these seven was diagnosed at postmortem examination. Twenty-five of the Asian elephants and 15 of the African elephants were considered noninfected. The other 19 Asian elephants and 11 African elephants had been exposed to one or more infected

Table 1. *Mycobacterium tuberculosis* culture status of three elephant herds.

Herd	Elephant species	Positive	Negative
A	African	0	2
	Asian	1	15
B	African	0	2
	Asian	2	2
E	African	0	6
	Asian	1	3

animals. Infected and exposed elephants were from five herds in Arkansas, California, Florida, and Illinois; negative animals were from California, Colorado, the District of Columbia, Indiana, Kentucky, Louisiana, Texas, and Washington.

The seroreactivities of the 30 exposed elephants, as well as those of four of the infected elephants, were followed over time. These animals were from three herds, labeled A, B, and E, with one, two, and one culture-positive elephants, respectively, in each herd (Table 1). Many of the animals in these herds underwent treatment during the course of this investigation. Treatment dosage and route of administration varied by institution, by elephant, and over time (S. K. Mikota, unpubl. data), and treatment regimes are not reported here.

Herd A contained one culture-positive female Asian elephant and 17 culture-negative elephants (including two female African elephants, one male Asian elephant, and 14 female Asian elephants). Serum samples and trunk washes were collected in October 1996 and then in December 1996, August 1997, January 1998, and December 1998. The positive cultures from this herd were from a 46-yr-old Asian female (A3) in October 1996 and December 1996. Intradermal tuberculin testing was performed immediately after serum collection in October 1996. Treatment with isoniazid, pyrazinamide, and rifampin was initiated for all elephants after serum sampling in December 1996; treatment was continued until August 1997 for the culture-negative animals and was continued until December 1997 for the culture-positive elephant.

Herd B consisted of three female Asian elephants, one male Asian elephant, and two female African elephants. In March 1997, a 33-yr-old female Asian elephant (B1) died and *M. tuberculosis* was cultured at postmortem. Stored serum samples from elephant B1, as well as stored and prospective serum samples from the other elephants in the herd, were analyzed. A 30-yr-old female Asian elephant (B6) from the herd was culture positive in March 1997. Elephant B6 was transported to a different

facility in April 1997, and treatment with isoniazid, rifampin, pyrazinamide, and pyridoxine was administered from May 1997 to December 1997. Elephant B6 was culture negative in October 1996, April 1997, May 1997, and October 1997. Treatment with isoniazid, rifampin, and pyrazinamide was administered to B2, a 39-yr-old Asian female, and B3, a 35-yr-old Asian male, from July 1997 to December 1997. Follow-up cultures, obtained every 1–2 mo, were negative.

Herd E contained one culture-positive elephant and nine exposed, culture-negative elephants. Four were male African elephants, two were female African elephants, and three were female Asian elephants. The infected elephant was a 10-yr-old male Asian elephant; all others in the herd were ≥ 15 yr old. At the time of initial serum sampling, six of the nine noninfected elephants (E2–E7) were culture positive for *M. avium* and/or other nontuberculosis complex mycobacteria. Intradermal tuberculin testing with balanced *M. bovis* PPD and *M. avium* PPD was conducted on all elephants in the herd immediately after initial serum sampling. At that time, the male was isolated from the rest of the herd and was started on a 12-mo course of treatment with isoniazid and pyrazinamide. Five months later, in March 1999, serum samples and trunk washes were collected from all of these animals. No elephant in herd E was *M. tuberculosis* culture positive at the time of the March 1999 sampling; however, *M. avium* was cultured from trunk washes of a 41-yr-old female Asian elephant (E2) and a 15-yr-old male African elephant (E5).

Data analysis

The OD values from each antigen were compiled and tested for normality by Shapiro and Wilks test.⁷ Antigen ODs that were not normally distributed were transformed using the Box–Cox method to determine transformation type.⁴ The transformed variables were then tested to assess the improvement in the normality of distribution. All variables were normally distributed after transformation.

Statistical analyses used BMPD/Dynamic Statistical Software (SPSS, Inc., 444 North Michigan Avenue, Chicago, Illinois 60611, USA).⁷ For each antigen, two-sample *t*-tests were used to determine significant differences in OD values between infected and noninfected elephants and between noninfected Asian and noninfected African elephants. Two-sample *t*-tests were also used to determine significant differences in ages for infected and noninfected elephants. Pearson correlation coefficients were used to determine the level of correlation between antigens.

As previously described,¹¹ discriminant analysis was used to determine the combination of antigens that most accurately predicted infection status of the most animals. The resulting classification functions were then used to calculate specificity and sensitivity. OD values obtained from ratios to 1:100 positive controls were analyzed separately from OD values obtained from ratios to 1:400 positive controls. The combination of antigens with the highest combined specificity and sensitivity was used to calculate positive and negative predictive values (PV⁺ and PV⁻, respectively). Confidence limits for sensitivity, specificity, PV⁺, and PV⁻ were calculated using a binomial model.¹ The level of significance for all test statistics calculated in the investigation was $\alpha = 0.05$.

RESULTS

The mean ages of infected (29.1 ± 7.9 yr, range = 10–46 yr) and noninfected elephants (26.6 ± 3.5 yr, range = 5–59 yr) were not significantly different.

The Shapiro–Wilks test for normality indicated that the antigens CF, PPD, and AVPPD were approximately normally distributed, whereas MPB, ERD, and RA were not. Box–Cox transformation methods suggested square root transformations for the latter three variables. The new variables were termed SMPB, SERD, and SRA.

Two-sample *t*-tests determined significant differences in OD values between infected and noninfected elephants. Significant differences in means were detected for CF, PPD, ERD (SERD), RA (SRA), and AVPPD; no significant difference in means was detected for MPB (SMPB) (Table 2).

Significant differences were detected in mean ODs between noninfected Asian elephants and noninfected African elephants. Significant differences were detected for CF, PPD, ERD (SERD), and AVPPD; no significant differences were detected in MPB (SMPB) nor RA (SRA) (Table 3).

Discriminant analysis indicated that the OD values obtained by calculating ratios to the 1:400 positive controls offered the best discriminative function for determining infection status. For clarity, only the OD values from the 1:400 positive control ratios are reported. Classification functions for determining elephant infection status were the probability that an animal was infected = $-35.48 + 82.13$ (CF) – 19.70 (SRA) and the probability that an animal was not infected = $-1.18 + 3.03$ (CF) + 3.39 (SRA). Specificity and sensitivity were both 100% (91.9% – 100% and 54.4% – 100%, respectively). A graphic representation of this discrimination is shown in Figure 1. Specificities and

Table 2. Optical density values and standard deviations, by ELISA for multiple antigens, for *Mycobacterium tuberculosis*-infected and noninfected elephants.

Antigen ^a	Infected (n = 7)	Noninfected (n = 40)
CF*	1.08 (±0.18)	0.09 (±0.15)
PPD*	0.91 (±0.29)	0.09 (±0.16)
MPB	0.15 (±0.29)	0.0 (±0.02)
ERD*	0.76 (±0.51)	0.20 (±0.32)
RA*	1.14 (±0.84)	0.26 (±0.49)
AVPPD*	0.86 (±0.29)	0.40 (±0.30) ^b

^a CF = culture filtrate of AN5 *Mycobacterium bovis*; PPD = purified protein derivative of *M. bovis*; MPB70 = *M. bovis* modified protein 70; ERD = lipoarabinomannan antigen Erdman strain *M. tuberculosis*; RA = lipoarabinomannan antigen H37 Ra strain *M. tuberculosis*; AVPPD = purified protein derivative of *Mycobacterium avium*.

^b AVPPD was not measured with one sample so n = 39 rather than 40.

* Statistically significant (P = 0.05) difference between infected and noninfected elephants.

sensitivities for each variable are presented in Table 4. The estimates of the positive and negative predictive value were both 100% (59.0% – 100% and 90.7% – 100%, respectively).

The correlation coefficient for CF and PPD was 0.971, and the correlation coefficient for ERD and RA was 0.884. All other correlation coefficients were <0.8 and are not reported.

The specificity of CF was 95.0% (89.2% – 100%). Using CF alone, the estimated positive predictive value was 77.8% (32.4% – 95.1%); the estimated negative predictive value was 100% (90.7% – 100%). With the discriminant equation for CF alone, a “cutoff” value of 0.58 was determined. This cutoff value correctly classified all but two of the 47 elephants used in the discriminant analysis. The cutoff value was used to evaluate the seroreactivity of 34 elephants over time. Elephants with ODs higher than the cutoff value were designated “ELISA positive.”

Herd A (Fig. 2)

The culture-positive female (A3) was ELISA positive in October 1996, ELISA positive with increased seroreactivity in December 1996, ELISA negative in August 1997 and January 1998, and ELISA positive again in December 1998. A 34-yr-old female Asian elephant (A4) was ELISA positive throughout sampling, with an increase in seroreactivity between October 1996 and December 1996. Although this animal was ELISA positive throughout treatment, its seroreactivity declined during treatment and then increased in December 1998. A 41-yr-old female Asian elephant (A14) was

Table 3. Optical density values and standard deviations, by ELISA for multiple antigens, for Asian elephants and African elephants noninfected with *Mycobacterium tuberculosis*.

Antigen ^a	Asian elephants (25)	African elephants (15)
CF*	0.12 (±0.18)	0.02 (±0.04)
PPD*	0.12 (±0.20)	0.03 (±0.06)
MPB	0.00 (±0.02)	0.0 (±0.00)
ERD*	0.29 (±0.38)	0.04 (±0.06)
RA	0.35 (±0.59)	0.11 (±0.16)
AVPPD*	0.52 (±0.27) ^b	0.19 (±0.23)

^a CF = culture filtrate of AN5 *Mycobacterium bovis*; PPD = purified protein derivative of *M. bovis*; MPB70 = *M. bovis* modified protein 70; ERD = lipoarabinomannan antigen Erdman strain *M. tuberculosis*; RA = lipoarabinomannan antigen H37 Ra strain *M. tuberculosis*; AVPPD = purified protein derivative of *Mycobacterium avium*.

^b AVPPD was not measured with one sample so n = 24 rather than 25.

* Statistically significant (P = 0.05) difference between species.

ELISA negative at initial sampling but was positive in December 1996 and remained positive throughout treatment. A 30-yr-old Asian female (A12) was ELISA negative at initial sampling, but seroreactivity increased moderately after i.d. tuberculin testing. This animal was still ELISA negative in December 1996 but was positive in all subsequent samplings. A 37-yr-old Asian female (A13) and a 24-yr-old Asian female (A18) remained ELISA negative except for the final samples in December 1998. Three Asian females (A8, A15, and A19) were ELISA negative at initial sampling in October 1996, positive in December 1996, and negative again through the remaining samples. The other elephants in the herd were always ELISA negative.

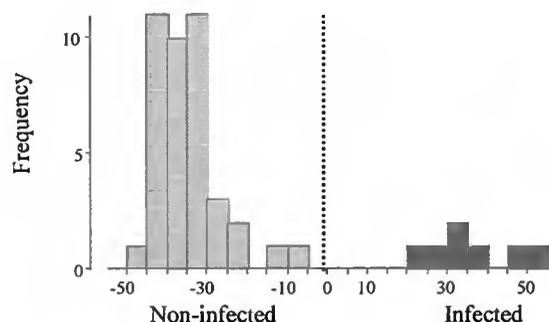
**Figure 1.** Graphic representation of multiple-antigen discriminant analysis model that differentiates *Mycobacterium tuberculosis*-infected from noninfected elephants on the basis of ELISA seroreactivity. The value “0” represents the cut-off value in the model that differentiates positive and negative animals. Solid blocks indicate culture-positive elephants.

Table 4. Specificity and sensitivity (with 95% confidence intervals) of ELISA antigens for detection of *Mycobacterium tuberculosis* infection in 47 elephants.

Antigen(s) ^a	Specificity	Sensitivity
CF	95.0% (89.2–100%)	100.0% (54.5–100%)
PPD	97.5% (92.7–100%)	85.7% (59.8–100%)
MPB	97.5% (92.7–100%)	28.6% (0–62.1%)
ERD	85.0% (74.4–96.1%)	71.4% (37.9–100%)
RA	90.0% (80.7–99.3%)	57.1% (9.1–100%)
AVPPD	76.9% ^b (63.7–90.1%)	71.4% ^b (37.9–100%)
SMPB	97.5% (92.7–100%)	57.1% (9.1–100%)
SERD	80.0% (67.6–92.4%)	71.4% (37.9–100%)
SRA	82.5% (70.7–94.3%)	71.4% (37.9–100%)
CF + SRA	100.0% (91.9–100%)	100.0% (54.4–100%)

^a CF = culture filtrate of AN5 *Mycobacterium bovis*; PPD = purified protein derivative of *M. bovis*; MPB = *M. bovis* modified protein 70; ERD = lipoarabinomannan antigen Erdman strain *M. tuberculosis*; RA = lipoarabinomannan antigen H37 Ra strain *M. tuberculosis*; AVPPD = purified protein derivative of *Mycobacterium avium*; SMPB = square root (transformed value) of MPB antigen; SERD = square root (transformed value) of ERD antigen; SRA = square root (transformed value) of RA.

^b AVPPD was not measured with one sample so total number of elephants = 46.

The avian antigen was not used in the multiple-antigen ELISA panel on serum from this group in the December 1996 sampling date.

Herd B (Fig. 3)

Samples for elephant B1 were ELISA positive in February 1996, June 1996, and March 1997. Samples for elephant B2 in February 1997 and July 1997 were ELISA negative. In July 1997, a single serum sample from elephant B3, a 35-yr-old Asian male, was ELISA negative; no trunk wash could be performed on this animal. Samples for elephant B4 were ELISA positive in June 1996, negative in February 1997, and positive again in June 1997; a trunk wash culture taken in July 1997 was negative. In July 1997, elephant B5 was culture negative and ELISA negative.

Elephant B6 was ELISA positive in October 1996 and March 1997. Seroreactivity increased in April 1997, after the elephant had been transported, but it declined again in October 1997. The elephant was ELISA negative in October 1997, at which time treatment with isoniazid and rifampin was initiated, and it was ELISA negative and culture negative during treatment. Two months after completion of treatment, elephant B6 was culture and ELISA negative; 5 mo after treatment had been completed, elephant B6 was ELISA positive and *M. avium* was cultured from a trunk wash.

Herd E (Fig. 4)

At the time of initial serum sampling (October 1998), only the infected male elephant (E1) was ELISA positive. Five months later, in March 1999, elephant E1 was culture negative but still ELISA

positive and had a slight increase in overall seroreactivity. During the 5-mo span, the seroreactivity of elephant E2 had increased substantially, and this animal had become ELISA positive; elephant E5 showed a moderate increase in seroreactivity but was not classified as ELISA positive. No other elephant in herd E showed any notable seroreactivity.

DISCUSSION

Serologic assays are widely used for surveillance, health certification, and diagnostic testing in wildlife because they are cost effective and because samples are easy to handle and store. However, the results of assays that detect antibody tend to vary more between species than do those from assays that directly detect microbes. There may be variability in species' immune responsiveness and exposure to cross-reacting agents; some serologic assays require species-specific reagents. Thus, a test cannot be assumed to be valid for all species.¹⁶

ELISA testing for tuberculosis has been performed in several species using a non-species-specific protein A labeled with horseradish peroxidase.^{11,31} Proteins A and G are produced by certain strains of *Staphylococcus aureus*. Each of these proteins binds the Fc portion of immunoglobulins (chiefly immunoglobulin G) of several mammalian species, although the degree of affinity for each protein is species dependent.^{12,14,21,34} Enzyme-labeled protein A has been widely used in immunologic investigations, and there is evidence that it is useful as a general purpose reagent, capable of detecting antibodies to mycobacteria in various mammalian species.^{12,31}

In 1980, this technique was investigated with a

Elephant Herd A

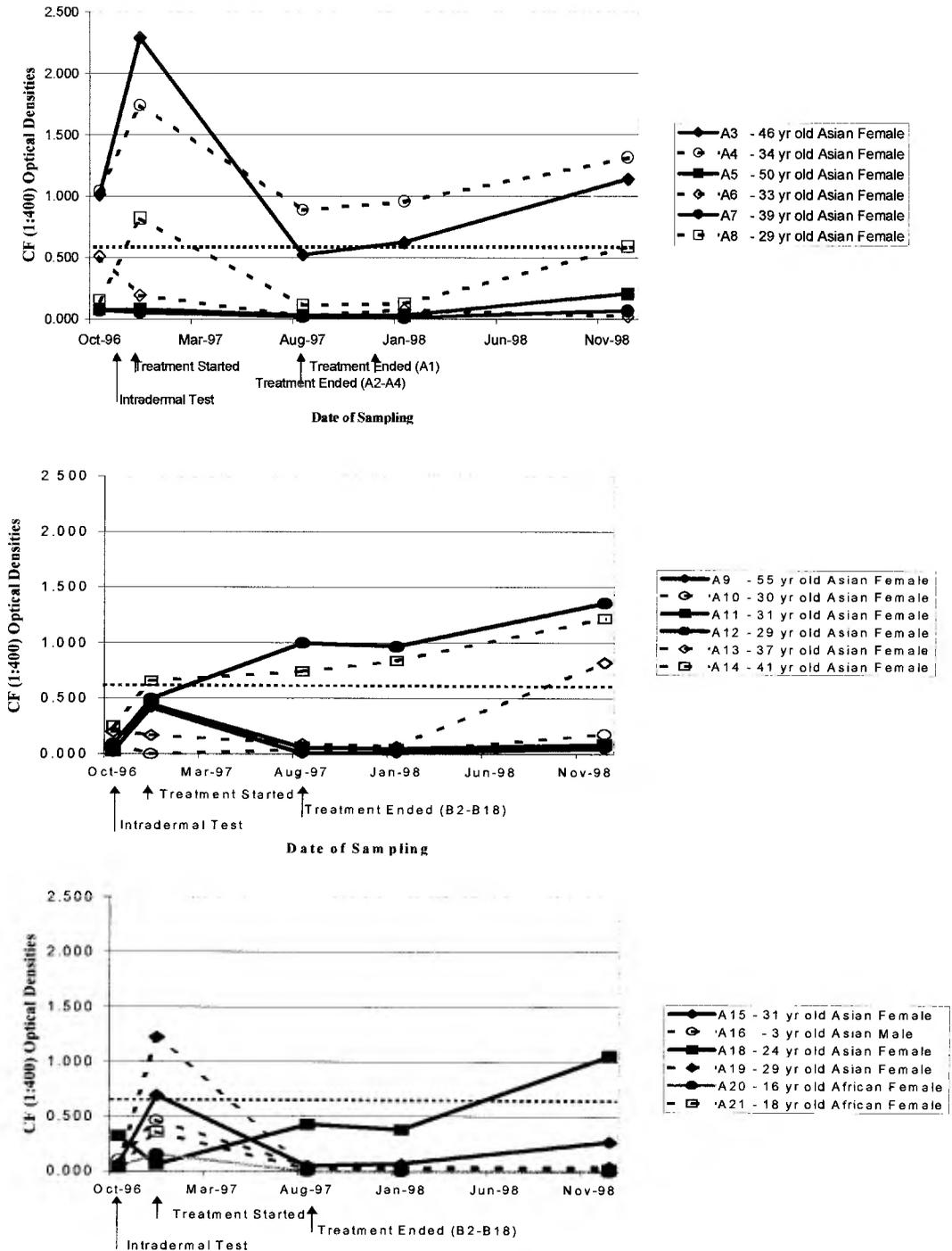


Figure 2. Optical density (OD) values of ELISA to *Mycobacterium bovis* AN5 culture filtrate (CF) for 18 elephants in herd A (elephants A3–A21). A female Asian elephant (A3) had trunk wash cultures positive for *Mycobacterium tuberculosis* in October 1996 and December 1996. No other elephant in the herd had a *M. tuberculosis*-positive trunk wash culture. - - - represents a “cut-off” value (OD = 0.58) for infected vs. noninfected as determined by discriminant analysis of CF.

Elephant Herd B

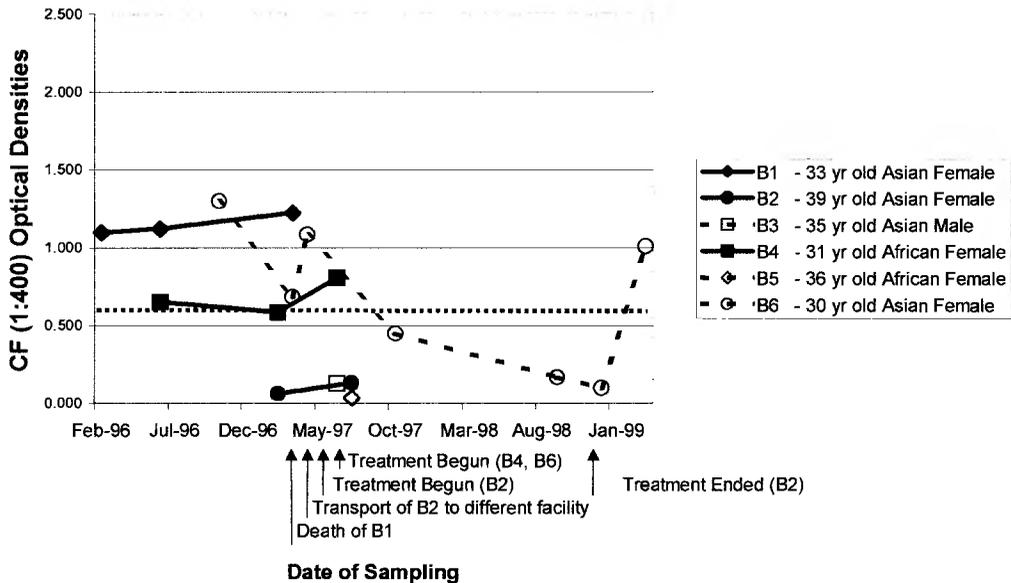


Figure 3. Optical density (OD) values of ELISA to *Mycobacterium bovis* AN5 culture filtrate (CF) for six elephants in herd B. Two female Asian elephants had cultures positive for *Mycobacterium tuberculosis*: elephant B1 was positive at postmortem exam in March 1997; elephant B6 had positive trunk wash cultures in March 1997, April 1997, and October 1997. Elephant B6 was transported to a different facility in April 1997 and had a positive trunk wash culture for *Mycobacterium avium* in March 1999. No other elephant in the herd had a positive trunk wash culture. - - - represents a “cut-off” value (OD = 0.58) for infected vs. noninfected as determined by discriminant analysis of CF.

herd of five elephants, one with a positive culture for *M. tuberculosis*,³¹ and antigens that included heat-killed cells of *M. bovis*, purified protein derivative of *M. bovis* (PPD), and heat-killed cells of *M. avium*. Two elephants that were i.d. tuberculin test positive showed substantial seroreactivity to heat-killed *M. bovis* and PPD, whereas two of three i.d. tuberculin test-negative animals showed seroreactivity to heat-killed *M. bovis* only. A subsequent investigation in 1998, with a herd of four presumed-negative elephants,²⁵ suggested that tuberculin exposure influences serologic analysis with ELISA and may affect the BTB test. The antigens used in this serologic investigation were *M. tuberculosis* CF protein, *M. avium* CF protein, *M. avium* sonicate antigen, PPD, avian purified protein derivative, and a lipoarabinomannan antigen. Elephants showed minimal to no reactivity to these antigens prior to i.d. injection but seroreactivity increased dramatically afterward. The BTB results were also affected because all elephants tested negative prior to antigenic stimulation, whereas two of three stimulated elephants tested positive for *M. bovis* antigen afterward. Elephants are constantly exposed to saprophytic *Mycobacteria* spp. and can be colonized

by atypical mycobacteria because of their behavior of dusting and bathing with their trunks. This exposure may account for nonspecific serologic reaction to mycobacterial antigens.²⁵

In this report, the significant differences in seroreactivity between infected and noninfected groups indicate a substantial humoral response in elephants with active *M. tuberculosis* infection. Between infected and noninfected groups, there were significant differences in the ODs for all antigens except MPB, and these differences do not appear to be associated with age-related exposure. It is not surprising that low reactivity to MPB was observed in both groups because MPB has been used to increase specificity for *M. bovis* screening in cattle and cervids.^{5,15,35} It is also not surprising that good specificity and sensitivity were achieved by a combination of antigens derived from *M. bovis* and *M. tuberculosis* because these mycobacteria are closely related, sharing 85–100% of homology at the DNA level.^{9,10} Shared antigenicity of these organisms has been documented.^{6,18,20}

This multiple-antigen ELISA was able to accurately discriminate between infected and noninfected elephants. The classification function that

Elephant Herd E

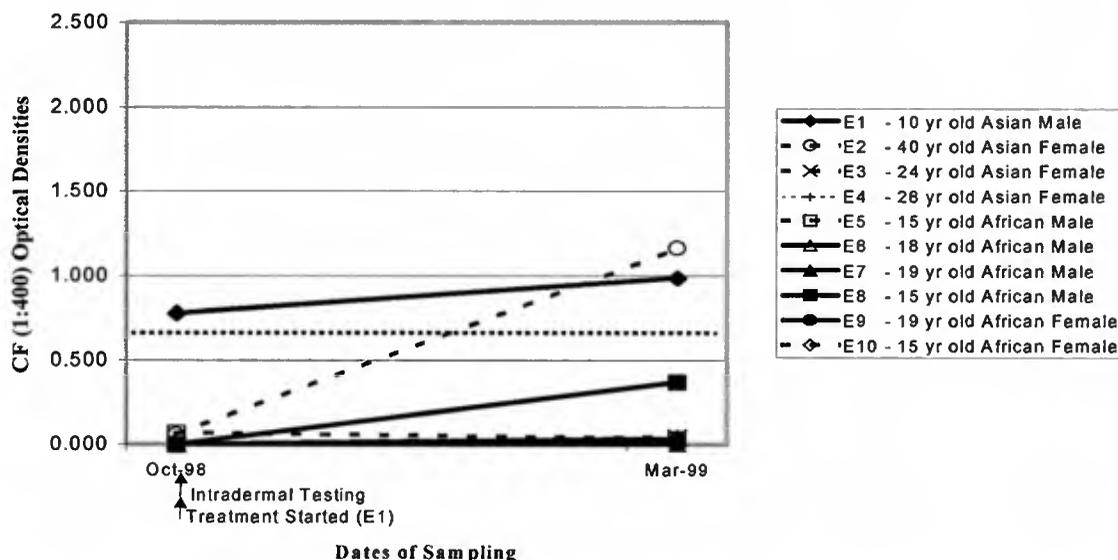


Figure 4. Optical density (OD) values of ELISA to *Mycobacterium bovis* AN5 culture filtrate (CF) for 10 elephants in herd E. A male Asian elephant (E1) had a *Mycobacterium tuberculosis*-positive trunk culture in October 1998; no other elephant in herd E had a *M. tuberculosis*-positive trunk culture. In October 1998, six elephants (E2–E7) had *Mycobacterium avium* or other nontuberculous mycobacteria cultured from trunk washes; in March 1999, elephants E2 and E5 had *M. avium* cultured. - - - represents a “cut-off” value (OD = 0.58) for infected vs. noninfected as determined by discriminant analysis of CF.

showed the best fit in discriminating infected from noninfected elephants included the variables CF and SRA. Although this allowed separation of the infected and noninfected groups with 100% accuracy, several caveats regarding these results should be noted. Although 100% specificity and 100% sensitivity were achieved with a small sample size (47), it is very likely that false negatives and false positives will occur with additional sampling. Furthermore, in other species, antibody titers increase with increased severity of disease, and ELISAs may have limited ability to detect early infections.⁵ Although high antibody levels were found in the positive elephants in this study, it is not known if antibody levels are elevated in *M. tuberculosis*-infected elephants that are not shedding bacteria.

A sensitivity of 100% and a 95% specificity were achieved with CF alone as a determinant of infection status. It is possible that the inclusion of RA in the model was artifactual and a result of small sample size because the exclusion of RA from the model only changed the total number of misclassified animals from 0 to 2. Assuming its inclusion in the model is not artifactual, the RA antigen appears to be useful in detecting nonspecific seroreactivity to mycobacterial antigens because its ad-

dition to the model increased specificity without decreasing sensitivity. With the discriminative function, elephants with high seroreactivity to CF or high seroreactivity to CF and RA are classified as infected; animals with high seroreactivity to RA and low seroreactivity to CF are classified as noninfected. Elephants with moderate levels of seroreactivity to CF and high levels of seroreactivity to RA are also classified as noninfected.

Neither PPD nor ERD was included in the discriminant analysis model even though the OD values for these were significantly different antigens between the infected and noninfected groups (Table 1). The absence of these antigens was due to a high correlation of PPD with CF and of ERD with RA. With their high correlations to variables already in the model, PPD and ERD did not add significant discriminating power to the model. These correlations were probably due to similarities in antigenic presentation.

Also, AVPPD was not added to the model. Although there was a significant difference between the mean values for infected and noninfected groups, extensive overlap in the ranges of OD values for these two groups (Table 2) prevented

AVPDD from being useful in discriminatory analysis.

The large confidence interval range for the positive predictive value is disconcerting, but expected, given the small number of infected elephants in this study. Tuberculosis prevalence in elephants in North America was estimated recently to be 3.0% on the basis of a survey of multiple zoos, circuses, government organizations, and the North American Regional Studbook Keepers for elephants (S. K. Mikota et al., unpubl.). This highly conservative estimate uses the total number of known culture-positive elephants (17) and an estimate of the total elephant population in North America (560). The positive predictive value of the ELISA might increase if the true prevalence of *M. tuberculosis* in the North American elephant population is higher than current estimates. However, if current estimates are accurate, the high negative predictive value is encouraging because few infected elephants will be misdiagnosed. A high negative predictive value is particularly important in disease management because undetected infections could spread to susceptible individuals.

The seroreactivity of noninfected African elephants and noninfected Asian elephants to CF, PPD, ERD, and AVPPD differed significantly, possibly because of inherently different seroreactivity, different exposure levels, the presence of culture-negative infected animals, and/or species differences in immune function. Without additional research, the cause of these differences will remain unknown. There are few documented cases of *M. tuberculosis* infection in African elephants, and whether this paucity is due to decreased susceptibility, decreased exposure, inadequate diagnostic testing, or other factors is unknown.

These results also imply that it may be inappropriate to combine results from Asian elephants and African elephants. In the future, each species should be individually evaluated. Although this would reduce sampled population size and, consequently, would reduce sample size, single-species evaluation should facilitate more precise evaluation. Furthermore, it will be impossible to adequately assess the utility of the ELISA in African elephants unless more cases of *M. tuberculosis* infection occur in this species.

Evaluation of the three elephant herds over time proved interesting. Many animals in herds A and E had marked increases in seroreactivity to CF after i.d. tuberculin testing; within each herd, there was considerable variation in serologic response to i.d. stimulation (Figs. 2, 4). These results suggest that there may be a maximal effect from i.d. tuberculin

testing occurring sometime between 2 wk and 3 mo postinjection. It is unclear whether this seroreactivity is due to previous infection, to nonspecific cross-reaction to other antigens, or to stimulation of humoral response in infected animals. Only by continued monitoring of these animals can the reasons for this variation be elucidated. These increases in seroreactivity suggest that false-positive reactions may occur after i.d. testing and that different evaluations of this test may be necessary depending on the timing of i.d. tuberculin testing relative to serum sampling.

The general decrease in seroreactivity for ELISA-positive animals during the course of treatment is notable. In herds A and B, CF values declined in many animals over the course of treatment (Figs. 2, 3) and then increased after treatment was completed. Infected elephant B6 followed this trend and had trunk wash cultures positive for *M. avium* on March 1999 (Fig. 3). This decline in seroreactivity may be due to decreased exposure of elephants to noninfectious mycobacteria as a result of the antimycobacterial drugs. The decline may also represent decreased numbers of *M. tuberculosis* organisms during treatment without successful eradication. Thus, a rise in seroreactivity after treatment may imply either reemergence of, or reinfection with, *M. tuberculosis*. In contrast to herds A and B, the infected animal in herd E had increased seroreactivity during treatment. This rise suggests either a lack of test specificity or inadequate treatment.

CONCLUSIONS

Arguments have been made against the use of serologic assays for detecting tuberculosis because of inadequate sensitivity, as the immune system response to mycobacteria is primarily cell mediated and high levels of circulating antibody are thought to indicate a failure of the immune response to contain mycobacterial growth and progression of disease.^{27,28} These arguments have not been dispelled by this investigation. Only animals that were actively shedding *M. tuberculosis* were used as confirmed positives, and the degree of seroreactivity in nonclinical, nonshedding, infected elephants is unknown. This will continue to be a problem in evaluating testing methods for elephants. Asian elephants and African elephants individuals are rare and long lived, so opportunities for postmortem sampling (as is done in evaluating tuberculosis testing in domestic cattle and captive cervids) occur infrequently.

Further investigation is clearly needed to evaluate the true utility of this test. Future investigations should include paired sampling of elephants before

and after i.d. tuberculin testing.²⁵ It remains to be determined whether i.d. testing would augment the ELISA by stimulating a humoral response in infected animals or if it would confound the ELISA by stimulating antibody production in animals currently or previously infected with nontuberculous mycobacteria.

The antigens with the greatest discriminatory power in this investigation were CF and RA. However, this does not mean that the other antigens in this panel lacked merit. Both MPB and AVPPD may be useful for differentiating *M. tuberculosis* infection from *M. bovis* and/or *M. avium* infection. Although these latter agents have not been well documented in elephants, they have infective potential and might not be detected by the discriminant function determined in this investigation. In contrast, PPD and ERD antigens may not be as important in future ELISA investigations because of their high correlation with other more specific and more sensitive antigens. It may be prudent to more extensively investigate CF, RA, MPB, and AVPPD as well as other antigens that are under development.

Acknowledgments: This investigation received financial support from the U.S. Department of Agriculture, Animal and Plant Health Inspection Service, Veterinary Services. We thank the veterinarians and animal care staff of the zoos and private institutions that contributed to this investigation. This work was submitted to the academic faculty of Colorado State University in partial fulfillment of the requirements for the degree of Master of Science.

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Received for publication 20 October 1999