Denise M Sofranko/NM/APHIS/USDA
11/01/2010 01:46 PM

To Barbara A Kohn/MD/APHIS/USDA
cc
bcc
Subject Fw: Another draft--response needed

Hey Barb,
Just to refresh some memories, here is an e-mail string from 2008 discussing the rationale for restrictions on the Stat-Pak. I have another one I'll send next that has more discussion on the subject.

----- Forwarded by Denise M Sofranko/NM/APHIS/USDA on 11/01/2010 11:45 AM -----

Larry R Ludemann/IA/APHIS/USDA
03/20/2008 03:01 PM

To Mandi M Frederick/MD/APHIS/USDA@USDA
cc Chester A Gipson/MD/APHIS/USDA@USDA, Denise M Sofranko/NM/APHIS/USDA@USDA
Subject Re: Another draft--response needed

I have no comments. Good job.

Larry Ludemann
Senior Staff Veterinarian
Center for Veterinary Biologics-Policy, Evaluation, Licensing
Mandi M Frederick/MD/APHIS/USDA

Mandi M Frederick/MD/APHIS/USDA
03/20/2008 03:36 PM

To Denise M Sofranko/NM/APHIS/USDA@USDA, Chester A Gipson/MD/APHIS/USDA@USDA, Larry R Ludemann/IA/APHIS/USDA@USDA
cc
Subject Another draft--response needed

Hi all,

Sorry to pester you again with this but my editor had a few changes to the Chembio letter. Please take another quick look and let me know if this works for you.

Thank you so much.

Mandi

Mandi M. Frederick
Writer/Editor
Executive Communications
Legislative and Public Affairs
4700 River Road, Unit 49
Riverdale, Maryland 20737
(301)734-0593
2A 02.30

Dear (b)(6) [redacted]

Thank you for your letters of February 27, 2008, to Secretary Ed Schafer and other officials at the Department of Agriculture (USDA), including USDA's Animal and Plant Health Inspection (APHIS) Administrator Cindy Smith, regarding USDA restrictions on the distribution of Chembio's Elephant TB STAT-PAK® Assay.

We recognize your interest in this issue and appreciate the opportunity to address your concerns. As you are aware, APHIS is responsible for enforcing the Animal Welfare Act, as well as safeguarding the health of America's agricultural animals. We assure you that we take these responsibilities very seriously and work hard to protect the health and well-being of all animals that fall under our purview.

USDA welcomes advances in testing technologies for significant animal diseases. While we believe that Chembio's test kit has legitimate and potentially valuable applications within the veterinary community, tuberculosis (TB) testing on domestic animals is currently permitted only in USDA-approved laboratories, in order to ensure test validity and to prevent fraud. It is necessary that USDA prevent misuse or abuse of any test kit—including Chembio's—that has the potential to be used on domestic species. Fraudulent TB test results could affect the movement of TB-exposed or infected animals in the United States, disrupting USDA's TB control efforts and dramatically impacting the health of the Nation's agricultural animals.

We look forward to receiving and reviewing the United States Animal Health Association's

recommendations on the care and treatment of TB in elephants, including any updated recommendations regarding TB testing. Contingent on the outcome of our review, we may take steps to revise our protocols regarding the care of TB-exposed and infected elephants. In the meantime, APHIS officials continue to discuss the restrictions on Chembio's test kit and the best way to maximize the kit's potential benefits while protecting the health of domestic animals.

We recognize Chembio's contributions to the health of the Nation's animals, and appreciate your patience as we continue to work on this issue.

Sincerely,

Denise M Sofranko/NM/APHIS/USDA
11/01/2010 09:57 AM

To Barbara A Kohn/MD/APHIS/USDA
cc
bcc
Subject Fw: Copy of the license for your information and the call on Tuesday

Have you seen this?

Denise M. Sofranko, DVM
Field Specialist for Elephants
USDA/APHIS/Animal Care
----- Forwarded by Denise M Sofranko/NM/APHIS/USDA on 11/01/2010 07:56 AM -----

Robert M Gibbens/CO/APHIS/USDA
11/01/2010 05:39 AM

To Ray M Flynn/CO/APHIS/USDA@USDA, "Denise M Sofranko" <Denise.M.Sofranko@aphis.usda.gov>
cc
Subject Fw: Copy of the license for your information and the call on Tuesday

Fyi

Chester A Gipson

----- Original Message -----
From: Chester A Gipson
Sent: 11/01/2010 06:33 AM EDT
To: Kay Carter-Corker; Andrea Morgan
Cc: Robert Gibbens; Betty Goldentyer; Nora Wineland; Allan Hogue
Subject: Fw: Copy of the license for your information and the call on Tuesday

Hi all,
I will be discontinuing the requirement for AC to look over the shoulder of individuals conducting the TB testing. An index of suspicion needs developing for situations where fraudulent sampling is suspected so we can use the existing fraudulent blood sampling program.
While I appreciate Lila's research of the FOIA issue, my position remains unchanged. I want a letter for my signature transmitting all pictures in our possession that Ringling has compensated chembio for. If memory serves me correctly all test except one has been paid for. I would like this completed by the time I return from travel next week (this includes the letter advising chembio that we will no longer be the intermediary for reporting test results to facilities if it has not been prepared) .
Thanks,
C. Gipson
301 734 4980
----- Forwarded by Chester A Gipson/MD/APHIS/USDA on 11/01/2010 06:15 AM -----

Rick E Hill/IA/APHIS/USDA
10/29/2010 10:27 PM

To Chester A Gipson/MD/APHIS/USDA@USDA, Elizabeth A Lautner/IA/APHIS/USDA@USDA
cc
Subject Copy of the license for your information and the call on Tuesday

Mycobacterium Bovis-Mycobacterium Tuberculosis Antibody Test Kit, Code 5470.01, produced by Chembio Diagnostic Systems, Inc., Est. No. 645

[attachment "645-547001-070815_license.pdf" deleted by Barbara A Kohn/MD/APHIS/USDA]

COPY

United States Department of Agriculture

UNITED STATES VETERINARY BIOLOGICAL PRODUCT LICENSE

Washington, D.C.,

This is to certify that, pursuant to the terms of the Act of Congress approved March 4, 1913 (37 Stat. 832), governing the preparation, sale, barter, exchange, shipment, and importation of viruses, serums, toxins, and analogous products intended for use in the treatment of domestic animals, the person holding United States Veterinary Biologics Establishment License No. **645** *is authorized to prepare in the facilities designated in the establishment license*

MYCOBACTERIUM BOVIS-MYCOBACTERIUM TUBERCULOSIS ANTIBODY TEST KIT

Code 5470.01

Preparation shall be in accordance with the provisions of the Act, the regulations made thereunder, and additional restrictions or requirements when listed below.

1. **For use by or under the supervision of a veterinarian.**
2. **Sale and use in the United States restricted to laboratories approved by state and federal (USDA) animal health officials.**

This license is subject to termination as provided in the regulations made under the authority contained in said Act, and to suspension or revocation if the licensee violates or fails to comply with said Act or the regulations made thereunder.

August 15, 2007
Date

Director, Center for Veterinary Biologics
Animal and Plant Health Inspection Service

APHIS FORM 2004A
(JUNE 97)

Previous edition may be used.

CERTIFIED—RETURN
RECEIPT REQUESTED

AUG 15 2007

(b)(6) [redacted]

Chembio Diagnostic Systems, Inc.
3661 Horseblock Road
Medford, NY 11763

Dear (b)(6) [redacted]

Enclosed is a new United States Veterinary Biological Product License issued this date to Chembio Diagnostic Systems, Inc., Establishment License No. 645, authorizing production of the following:

Mycobacterium Bovis-Mycobacterium Tuberculosis Antibody Test Kit,
Code 5470.01

Please note the restrictions under which this license is issued.

This U.S. Veterinary Biological Product License does not constitute a patent license. If this product or technology used in the manufacture of this product has been patented or is pending patent, the licensee should obtain a patent license from the patent owner.

If this license does not agree with your records, please return it to this office with your comments.

Sincerely,

/s/ Richard E. Hill, Jr.

Richard E. Hill, Jr.
Director
Center for Veterinary Biologics

Enclosure

cc:
CVB-IC
Master File
B. J. Erdahl

APHIS:VS:CVB-PEL:LRLudemann:ah:232-5785:8-6-07
W:\FirmsCorrespondence\645\Corresp\645_ProdLic_5470.01_070806_Ltr.doc

Larry Ludemann
RTS 8-6-07
FPD 8-15-07

A.H.
65511

NEW PRODUCT LICENSE SUBMITTED FOR DIRECTOR'S APPROVAL

Establishment No. 645 Product License No. 5470.01 Mycobacterium Bovis-Mycobacterium Tuberculosis Antibody Test Kit

Legal Instrument Examiner (LIE) or Program Assistant (PA) brings license to desk for Director's signature. At this point, the license has been checked several times to ensure it is error free. There is a checklist in the front of the license folder that has been filled out by the PA and initialed by the LIE. There is a place for PSA's initials on the form, and this is done after one more check for any possible errors. Following are some of the items to check before giving to the Director, PEL.

BJL 8-9-07 The blocks on the License checklist are filled in as completely as possible. There are some items in the PA's section that cannot be filled in until after the license is signed.

BJL 8-9-07 The PA has initialed the checklist.

BJL 8-9-07 Read through the license carefully checking for any misspelled or missing words.

BJL 8-9-07 Read through the transmittal letter carefully checking for any misspelled or missing words.

BJL 8-9-07 Ensure letter has the following signature block:

Richard E. Hill, Jr.
Director
Center for Veterinary Biologics

✓ The packet contains the following items in the following order:

BJL 8-9-07 Original of license letter

BJL 8-9-07 Product License (Reviewer and Section Leader's initials should appear in border of License.)

BJL 8-9-07 Application for license (2003 for Product License)

BJL 8-9-07 Labels with 2015 (should be signed by Reviewer but will not have label numbers yet.)

BJL 8-9-07 Outline of Production

BJL 8-9-07 No. 1 (historical) file. This file has a red cover and is pegged at the top. There may be other volumes for immediate archival included in the license packet.

BJL 8-9-07 Colored copies of transmittal letter with Reviewer and Section Leader's initials above the legend line on the yellow copy.

NOTE: There may be a green folder attached that contains some materials such as previously approved sketches. This folder is placed on the bottom of the stack of license materials.

BJL 8-9-07 Initial on the license checklist.

BJL 8-9-07 Initial in the lower left corner of the yellow copy of the transmittal letter.

BJL 8-9-07 Make notation in "CVB Notice Log (Project & Docket Lists) and License Approval Log Book" (hereafter called "Log Book") located in the overhead bin beside your computer, as to date, Establishment Number, Product Code, True Name. The log sheet in the Log Book is self-explanatory.

BJL 8-9-07 Place all materials in Director, PEL's in-box. The Director, PEL may or may not sign the license for the Director, CVB. If he signs the license and transmittal letter, note in the Log Book the date signed and the number of days elapsed since submitted to Director, PEL for signature. If he sends it on to Director, CVB, make notation in Log Book as to date it was forwarded.

BJL 8-15-07 When license and transmittal letter are signed, record date signed and number of days elapsed since submitted to Director, PEL for signature in the Log Book. Give entire license packet to designated LIE for one final check before PA dates materials.

RMS 4/11/07

This application must be submitted for issuance of a U.S. Veterinary Biological Product License. The application and attached supporting documents will be used for determining the purity, safety, potency, and efficacy of the product(9 CFR 102).

Public reporting burden for this collection of information is estimated to average 1.0 hours per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Department of Agriculture, Clearance Officer, OIRM, Room 404-W, Washington, D.C. 20250; and to the Office of Information and Regulatory Affairs, Office of Management and Budget, Washington, D.C. 20503.

U.S. DEPARTMENT OF AGRICULTURE
ANIMAL AND PLANT HEALTH INSPECTION SERVICE
VETERINARY SERVICES, CENTER FOR VETERINARY BIOLOGICS

FORM APPROVED - OMB NO. 0579-0013

APPLICATION FOR UNITED STATES VETERINARY BIOLOGICAL PRODUCT LICENSE

FOR VETERINARY BIOLOGICS USE ONLY
PRODUCT CODE NO. 5470.01

INSTRUCTIONS: Submit one copy for each product. If additional space is needed, attach additional sheets and refer to Item No. Enclose supporting documents.

1. ESTABLISHMENT LICENSE NO.
645

2. NAME AND ADDRESS OF APPLICANT (*Include No., Street, or RFD No., City, State, Zip Code*)
Chembio Diagnostic Systems, Inc.
3661 Horseblock Road
Medford, NY 11763

3. ADDRESS OF PREMISES TO BE USED (*If different from Item 2*)
Same as 2.

RCVD MAR 22 '07

4. BIOLOGICAL PRODUCT TRUE NAME
Mycobacterium bovis - Mycobacterium tuberculosis antibody test kit

5. HAS A LICENSE BEEN TERMINATED WHICH WAS ISSUED TO YOUR FIRM FOR THIS PRODUCT?
[X] NO [ ] YES (*If "yes," explain why*):

CHECKLIST FOR SUPPORTING MATERIAL

ITEMS SUBMITTED	A. WITH THIS APPLICATION ("X")	B. DATE OF PREVIOUS SUBMISSION	FOR VETERINARY BIOLOGICS USE ONLY
6. Outline of Production (*Submit with APHIS Form 2015*)			
7. Labels or Sketches (*Submit with APHIS Form 2015*)			
8. Blueprints, Plot Plans, and Legends (*Submit if not on file*)	x	Dec. 4, 2006	
9. Research Protocols and Data (*Specify*)			
a.			
b.			
c.			
d.			
e.			
f.			
10. Personnel Biographies (*APHIS Form 2007, submit unless previously sent*)			
11. Other (*Specify*)			

CERTIFICATION

In accordance with the Act of Congress approved March 4, 1913 (37 Stat. 832-833; 21 U.S.C. 151-158) application is hereby made for a license to prepare the herein named animal biological product for use in the treatment of domestic animals. If a license is issued under this application, the licensee expressly agrees to comply with the provisions of the said Act, and all rules, regulations, and orders of the Department issued pursuant thereto governing the preparation, testing, and distribution of the animal biological product, and that the animal biological product will not be labeled or advertised so as to mislead or deceive the purchaser in any particular.

In case a product license is issued, it is further agreed that the biological product shall be subject to any additional requirements or restrictions stated therein.

12. SIGNATURE OF AUTHORIZED OFFICIAL	13. TITLE	14. DATE SIGNED
(b)(6) [redacted]	VP Regulatory Affairs / QA	3/21/2007

FOR VETERINARY BIOLOGICS USE ONLY

15. DATE FINAL SUPPORTING MATERIAL RECEIVED	16. LICENSE APPROVED BY (*Signature*)	17. DATE APPROVED
Jun 20-07	Larry Eudemann	August 15, 2007

APHIS FORM 2003
OCT 2001

Previous editions are obsolete.

A.H. 65511

Barbara A Kohn/MD/APHIS/USDA
10/20/2010 07:56 AM

To Denise M Sofranko/NM/APHIS/USDA@USDA
cc Nora E Wineland/CO/APHIS/USDA@USDA
bcc
Subject Re: Questions/comments from Cindy Smith

Nora and Denise -

I, too, should be available anytime on Thursday (before 4 PM EDT)....Thanks.

Barb
Denise M Sofranko/NM/APHIS/USDA

Denise M Sofranko/NM/APHIS/USDA
10/19/2010 10:49 PM

To Nora E Wineland/CO/APHIS/USDA@USDA
cc Barbara A Kohn/MD/APHIS/USDA@USDA
Subject Re: Questions/comments from Cindy Smith

Hi Nora,
I imagine I'll be busy catching but will be available at your convenience. Anytime after 7 mountain time. I think our call with Ray is friday.
Just let me know what works best for you two.
Thanks!

Nora E Wineland---10/19/2010 06:44 AM MDT---

From: Nora E Wineland
To: Denise Sofranko
Cc: Barbara Kohn
Date: 10/19/2010 06:44 AM MDT
Subject: Re: Questions/comments from Cindy Smith

Denise --- Barb and I were on the call yesterday regarding how we will proceed to answer all of these questions. What does your Thursday look like for a call to catch up on things?? I am available at 9 am eastern time, or after 12:30 eastern. Let me know what will work on your end. Barb -- please do the same and let me know what times work for you.

Thanks,

Nora

****

Nora E Wineland, DVM MS DACVPM
Director, Center for Animal Welfare
USDA-APHIS-AC
6501 Beacon Drive
Kansas City, MO 64141
816-926-2130

Denise M Sofranko---10/18/2010 01:00:22 PM---Denise M Sofranko/NM/APHIS/USDA

Denise M Sofranko/NM/APHIS/USDA
10/18/2010 01:00 PM

To Chester A Gipson/MD/APHIS/USDA@USDA

cc Allan T Hogue/MD/APHIS/USDA@USDA, Andrea M Morgan/MD/APHIS/USDA@USDA, Barbara A Kohn/MD/APHIS/USDA@USDA, Betty J Goldentyer/NC/APHIS/USDA@USDA, James M Tuck/MD/APHIS/USDA@USDA, Kay A Carter-Corker/MD/APHIS/USDA@USDA, Nora E Wineland/CO/APHIS/USDA@USDA, Robert M Gibbens/CO/APHIS/USDA@USDA, Thay Ly/MD/APHIS/USDA@USDA, Yvette D Joyner/MD/APHIS/USDA@USDA

Subject Re: Questions/comments from Cindy Smith

Hi all,

I'm still on leave but checking in from time to time. I'll be back in my office on Thursday.

I will review and answer more of the attached questions if necessary when I'm back but I want to be very clear about one of them in particular.

As far as I know no one from AC has ever required that an elephant be treated on the basis of a MAPIA test. I have been telling people just the opposite. We are not making people treat elephants. There is a provision in the Guidelines for prophylactic treatment but that is a decision to be made by the attending veterinarian. This decision should be made on the basis of many factors, including age and medical condition of the elephant and exposure history.

We have put some attending veterinarians in touch with experts in this area to discuss but in the Dickerson Park case, the experts were consulted after treatment had been started.

I don't know how this rumor got started and I only know of the one case in Missouri (Dickerson Park). I don't know anything about the other states that are referenced in the questions.

I was also asked to answer a similar list of questions by (b)(6) on behalf of the EMA board when I was at the meeting earlier this month.

Thanks!

Denise M. Sofranko, DVM
Field Specialist for Elephants
USDA/APHIS/Animal Care

Chester A Gipson---10/18/2010 09:09:39 AM---Attached are questions/comments prepared by the administr

Chester A Gipson/MD/APHIS/USDA
10/18/2010 09:09 AM

To Kay A Carter-Corker/MD/APHIS/USDA@USDA, Robert M Gibbens/CO/APHIS/USDA@USDA, Betty J Goldentyer/NC/APHIS/USDA@USDA, Allan T Hogue/MD/APHIS/USDA@USDA, Thay Ly/MD/APHIS/USDA@USDA, Nora E Wineland/CO/APHIS/USDA@USDA, Yvette D Joyner/MD/APHIS/USDA@USDA, Andrea M Morgan/MD/APHIS/USDA@USDA, James M Tuck/MD/APHIS/USDA@USDA, Barbara A Kohn/MD/APHIS/USDA@USDA, Denise M Sofranko/NM/APHIS/USDA@USDA

cc

Subject Questions/comments from Cindy Smith

Attached are questions/comments prepared by the administrator from the meeting she attended with ringling representatives and the Office of the Secretary. The scanner was disoriented:>).
Thanks,
C. Gipson
301 734 4980
[attachment "Cindy TB Questions.pdf" deleted by Denise M Sofranko/NM/APHIS/USDA]

CATALOG # 60-9680-0
5 Test Kit

CHEMBIO
DIAGNOSTIC SYSTEMS, INC.

3661 Horseblock Road
Medford, New York 11763
U.S. Veterinary License No. 645

Mycobacterium bovis – Mycobacterium tuberculosis
Antibody Test Kit
ElephantTB STAT-PAK® Assay

A Rapid Immunochromatographic Test for the Detection of Antibodies to *Mycobacterium tuberculosis* and *Mycobacterium bovis* in Elephant Serum, Plasma or Whole Blood

FOR *IN VITRO* VETERINARY DIAGNOSTIC USE
READ INSTRUCTIONS FOR USE CAREFULLY BEFORE PERFORMING TEST

INTENDED USE

The ElephantTB STAT-PAK Assay is a qualitative, single use, two-step, immunochromatographic screening test for the detection of antibodies to *Mycobacterium tuberculosis* and *Mycobacterium bovis* in serum, plasma or whole blood from African elephants (*Loxodonta africana*) and Asian elephants (*Elephas maximus*). The test is used as an aid in the diagnosis of active tuberculosis (TB) in conjunction with other diagnostic methods.

If specific antibodies are present in the sample, the expected test result is reactive. A reactive result is suggestive of active TB. In the absence of antibodies, the expected test result is nonreactive.

SUMMARY AND EXPLANATION

Tuberculosis (TB) in elephants is a re-emerging zoonotic disease caused primarily by *Mycobacterium tuberculosis* and, in some cases, by *Mycobacterium bovis*. The only USDA-recommended diagnostic test for TB in elephants is mycobacterial culture of trunk wash samples. However, there is a growing body of evidence indicating that this method has poor sensitivity, as it can only identify animals with extensive shedding of the organism that typically occurs late in the course of disease. Rapid detection of infected elephants is a crucial prerequisite for more effective control of TB, as early diagnosis allows timely initiation of chemotherapy [1-3].

Serological methods constitute an attractive alternative as they are simple, inexpensive, relatively non-invasive, and they do not depend on detection of mycobacteria [4-5]. None of the existing TB tests alone is sufficient to diagnose disease. Therefore, new TB diagnostic algorithms are being developed, in which serological assays may play an important role (see PERFORMANCE CHARACTERISTICS below).

The Chembio ElephantTB STAT-PAK Assay is a rapid immunochromatographic test for antibody detection that is safe, simple, and easy to perform.

PRINCIPLE OF TEST

The Chembio ElephantTB STAT-PAK Assay is based on immunochromatographic (lateral-flow) technology. The test employs a unique cocktail of recombinant *M. tuberculosis* proteins that are bound to the membrane solid phase. Blue latex particles conjugated with protein are used as the detection system. The ElephantTB STAT-PAK Assay can be used with serum, plasma or whole blood. Once a test sample is applied to the SAMPLE (S) well followed by the addition of a diluent, it flows laterally through the membrane strip. When it reaches the conjugate pad, antibodies, if present, bind to protein-latex conjugate and then the migrating immune complex binds to the antigens on the solid phase in the TEST (T) area producing a blue line. In the absence of antibodies there is no line in the TEST (T) area. The sample continues to migrate along the membrane and produces a blue line in the CONTROL (C) area demonstrating that the reagents are functioning properly.

MATERIALS PROVIDED

Each kit contains the following items:

- 5 ElephantTB STAT-PAK test devices
- 5 Disposable pipettes
- 1 Diluent vial (5mL)
- 1 Product insert

Additional Material Required But Not Provided

- Clock, watch or other timing device
- Disposable gloves
- Biohazard disposal container
- Collection devices for specimens

STORAGE AND STABILITY

The ElephantTB STAT-PAK Assay should be stored at 8 to 30°C in the original sealed pouch. The diluent should be stored in the original vial at 8 to 30°C. The kit is stable until the date printed on the box label and/or pouch.

NOTE: Do not use expired test kits.

CAUTION: Do not freeze test kits.

PRECAUTIONS

1. The test is designed FOR *IN VITRO* DIAGNOSTIC USE only. Use the test only in accordance with instructions supplied with the kit.
2. Handle all specimens as recommended for any potentially infectious serum or blood specimen in the CDC-NIH manual, *Biosafety in Microbiological and Biomedical Laboratories, 4th ed., 1999*
3. Use suitable protective clothing (gloves, lab coat, safety glasses) when handling samples or test devices after samples have been applied. Avoid any contact between hands, eyes, nose or mouth during specimen collection and testing.
4. Do not pipette any material by mouth. Do not smoke, eat or drink in areas where specimens or kit material are kept.
5. All testing should be performed at a temperature of 18 to 30°C.
6. After the completion of the assay, carefully dispose of materials treating them as biohazardous waste.
7. Do not use expired test kits. Do not freeze test kits.
8. Do not mix reagents from different kit lots.

SPECIMEN COLLECTION

The ElephantTB STAT-PAK Assay can be performed on whole blood, serum or plasma.

Whole Blood: Collect whole blood into tubes containing heparin or EDTA. Be sure to thoroughly mix whole blood by inverting capped tube several times just prior to testing. Follow test procedure instructions.

Serum: Serum is used from whole blood collected aseptically by venipuncture into a clean tube without anticoagulant. Allow the blood to clot at room temperature, centrifuge at 2000 rpm for 10 minutes at room temperature, 18 to 30°C, and separate the serum from the clot.

Plasma: Collect whole blood with anticoagulant (heparin, EDTA or sodium citrate), centrifuge at 2000 rpm for 10 minutes at room temperature, 18 to 30°C, and isolate the plasma supernatant.

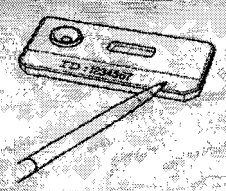
Samples perform best when tested immediately after collection. Specimens should be immediately refrigerated at 2 to 8°C following collection and can be used up to 3 days. If testing within 3 days is not possible, the specimens should be frozen at -20°C or colder until use. Avoid repeated freezing and thawing. DO NOT FREEZE WHOLE BLOOD.

NOTE: If specimens are to be shipped, they should be packed in compliance with regulations covering the transportation of etiologic agents. Venous whole blood, serum and plasma specimens should be shipped refrigerated with cold packs or wet ice.

TEST PROCEDURE

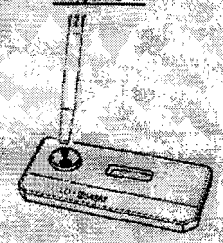
1. If test samples are refrigerated, remove them from the refrigerator and allow them to come to a temperature of 18 to 30°C before testing.
2. Remove the required number of ElephantTB STAT-PAK Assay devices from their pouches and place the devices on a flat surface area. It is not necessary to remove the desiccant from the package. **NOTE**: If desiccant packet is missing, DO NOT USE, discard the test device and a new test device should be used.
3. Label test units with sample names and/or identification numbers. (see Figure 1 below)

Figure 1



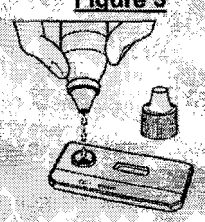
4. Using a disposable pipette, draw the specimen to be tested (whole blood, serum or plasma), into the pipette being careful not to draw up any air and add one full drop (30 µl) of specimen onto the center of the SAMPLE (S) well. (See Figure 2 below)

Figure 2



5. Once the specimen has been applied to the SAMPLE (S) well, remove the cap, invert the diluent bottle and hold it vertically (not at an angle) over the SAMPLE well. Add the diluent slowly dropwise; add **3 drops (~100 µl)** into SAMPLE (S) well. (See Figure 3)

Figure 3



6. Read results at **20 minutes** after the addition of diluent. **Do not read any results after 30 minutes.** Refer to INTERPRETATION OF RESULTS section below.
7. Discard the used disposable pipette, test device and any other test materials into a biohazard waste container.

QUALITY CONTROL

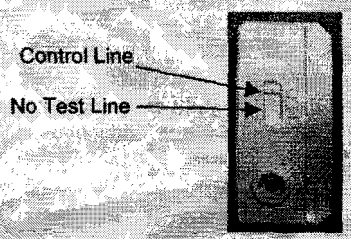
A blue colored line should always appear in CONTROL (C) area if the test has been performed correctly and the device is working properly. It serves as an internal test procedural control.

Good Laboratory Practice (GLP) recommends the use of control materials along with the test samples to ensure proper performance of the test kit. Positive and Negative serum or plasma based commercial controls should be used for this purpose. Use controls as per the TEST PROCEDURE instructions of this insert.

INTERPRETATION OF RESULTS

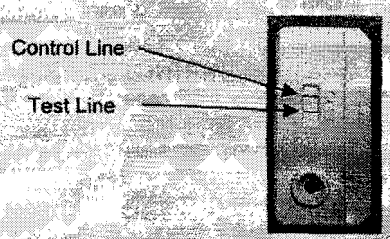
Nonreactive Result

One blue colored line in the CONTROL (C) area, with no visible colored line in the TEST (T) area indicates a nonreactive result. A nonreactive result at 20 minutes means that neither *Mycobacterium tuberculosis* nor *Mycobacterium bovis* antibodies were detected in the specimen. A nonreactive result does not preclude the possibility of TB infection.



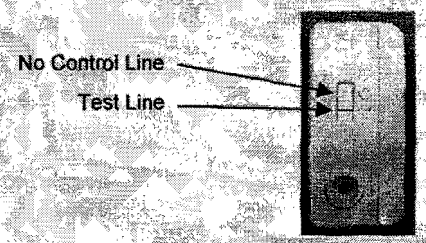
Reactive Result

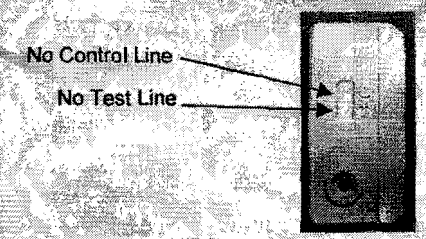
Two blue lines - one in the TEST (T) area and one in the CONTROL (C) area - indicate a reactive result. Intensities of the TEST and CONTROL lines may vary. Even a very faint line in the TEST (T) area of the device within 20 minutes is indicative of a reactive result. A reactive result means that *Mycobacterium tuberculosis* and/or *Mycobacterium bovis* antibodies were detected in the specimen



INVALID RESULTS

A blue line should always appear in the CONTROL (C) area, whether or not a line appears in the TEST (T) area. If there is no distinct blue line in the CONTROL (C) area, the test is invalid and should be repeated using a new device.





LIMITATIONS OF THE PROCEDURE

1. The assay is designed for detecting antibodies against *M. tuberculosis* and *M. bovis* only from elephant plasma, serum or whole blood. Any other body fluids or pooled samples or specimens from other than elephant species should not be used.
2. A reactive result suggests the presence of antibodies to *M. tuberculosis* and/or *M. bovis*.
3. For a reactive result, the intensity of the test line does not necessarily correlate with the titer of antibody in the specimen.
4. Reading nonreactive results earlier than 20 minutes or any results later than 30 minutes may yield erroneous results.
5. Do not use hemolyzed blood samples.
6. Blood specimens must be thoroughly mixed just prior to testing.
7. Be careful to add only 30 µL of specimen and 3 drops of diluent after applying the specimen to the SAMPLE (S) well.
8. Do not open the sealed test pouch until just prior to use.
9. Do not use kit contents beyond labeled expiration date.
10. Read results in a well-lit area.

PERFORMANCE CHARACTERISTICS

Highly specific and sensitive antibody binding proteins are used in the ElephantTB STAT-PAK Assay. The diagnostic performance was compared to the standard USDA-recommended method, trunk wash culture, and the ElephantTB STAT-PAK Assay was found to be superior.

Further, it was shown that both Asian and African elephants infected with either *M. tuberculosis* or *M. bovis* could be detected by ElephantTB STAT-PAK Assay up to several years prior to finding positive culture in trunk washes [3].

Sensitivity and Specificity

Sensitivity of the ElephantTB STAT-PAK Assay was determined by testing 23 culture positive elephants. Of these samples, 23/23 were reactive by the Chembio ElephantTB STAT-PAK antibody test kit (Table 1).
The specificity of the ElephantTB STAT-PAK Assay was determined by testing 131 serum, plasma, and/or whole blood samples. Of these samples 127/131 were non-reactive by the Chembio ElephantTB STAT-PAK antibody test kit (Table 2).

Table 1.
Diagnostic sensitivity of ElephantTB STAT-PAK Assay

Elephant	Mycobacterial species	ElephantTB STAT-PAK reactive
African	*M. tuberculosis*	3/3
African	*M. bovis*	1/1
Asian	*M. tuberculosis*	19/19

Table 2.
Specificity studies of ElephantTB STAT-PAK Assay

Elephant	ElephantTB STAT-PAK non-reactive	Trunk Lavage Culture negative
African	58/58	58/58
Asian	50/54	54/54
Unknown	19/19	19/19

REPRODUCIBILITY STUDIES

Reproducibility was tested at three independent laboratories using three serials of ElephantTB STAT-PAK Assay. A reference panel of 30 blinded samples representing negative, weakly reactive and reactive were tested 3 different times on 3 different days. The compiled results from 3 laboratories demonstrated 98.5% accuracy.

REFERENCES

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2. Montali, R.J., Mikota, S.K., and Cheng, L.I. (2001) *Mycobacterium tuberculosis* in in zoo and wildlife species. *Rev. sci. tech. Off. int.Epiz.* 20: 291-303.
3. Mikota, S.K., Peddie, L., Peddie, J., Isaza, R., Dunker, F., West, G., Lindsay, W., Larsen, R.S., Salman, M.D., Chatterjee, D., Payeur, J., Whipple, D., Thoen, C., Davis, D. S., Sedgwick, C., Montali, R.J., Ziccardi, M., and Maslow, J. (2001) Epidemiology and diagnosis *Mycobacterium tuberculosis* in captive Asian elephants (*Elephas maximus*). *J. Zoo Wildl. Med.* 32: 1-16.
4. Lyashchenko, K.P., et al., (2006) Tuberculosis in Elephants: Antibody Responses to Defined Antigens of *Mycobacterium tuberculosis*, Potential for Early Diagnosis, and Monitoring of Treatment. *Clinical And Vaccine Immunology* 13: 722-732.
5. Larsen, R.S., Salman, M.D., Mikota, S.K., Isaza, R., Montali, R.J., and Triantis, J. (2000) Evaluation of a Multiple-Antigen Enzyme-Linked Immunosorbent Assay for the Detection of *Mycobacterium Tuberculosis* Infection in Captive Elephants. *J. Zoo Wildl. Med.* 31: 291-302.
6. Lyashchenko K.P., Singh M., Colangeli R., and Gennaro M.L. (2000) A multi-antigen print immunoassay for the serological diagnosis of infectious diseases. *J. Immunol. Methods* 242: 91-100.

FOR MORE INFORMATION, CONTACT:

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3661 HORSEBLOCK ROAD
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U.S. Veterinary License No. 645
Tel: (631) 924-1135
Fax: (631) 924-6033
Email: info@chembio.com
Web Site: www.chembio.com

ORDERING INFORMATION

Cat #	Product
60-9680-0	ElephantTB STAT-PAK® 5 Test Kit
60-9682-0	ElephantTB STAT-PAK® 20 Test Kit
60-9683-0	ElephantTB STAT-PAK® 50 Test Kit

Antigens licensed from Statens Serum Institut
www.ssi.dk

AMERICAN ASSOCIATION OF ZOO VETERINARIANS
"Dedicated to wildlife health and conservation"
581705 White Oak Road • Yulee, Florida, 32097 • 904-225-3275 • Fax 904-225-3289

July 6, 2009

A. Kevin Shea
Acting Administrator USDA APHIS
1400 Independence Avenue, SW
Jamie L. Whitten Building, Room 312-E
Washington, DC 20250-3432

Dear Mr. Shea:

We would like to thank you for taking the time to meet with us on June 3 regarding our concerns about the new "Guidelines for the Control of Tuberculosis in Elephants" (Guidelines). We are writing in response to your request that we outline the concerns about the guidelines and their implementation. The AAZV values our relationship with the USDA and particularly the contribution our members make in the creation of recommendations regarding animal health issues. We appreciate the decision to delay implementation of the Guidelines and these new requirements in order to allow time for the USDA to address these issues and to allow questions regarding test interpretation and other matters to be addressed to the USAHA Committee on Tuberculosis.
As you requested at our meeting we have prepared a list of the primary areas of concerns in the hope that this will be the first step in the process of stakeholder consultation referenced in Dr. Gipson's most recent letter.

The specific items of concern are as follows:

- Accredited veterinarians recognize the importance of disease testing and our responsibility to maintain animal and public health. We feel that the requirement for federal oversight of specimen collection and shipping is unnecessary and will make testing difficult as it would require us to limit our specimen collection based on the availability of APHIS Animal Care staff.
- As noted during our meeting, Chembio also produces a Stat-Pak® for primates that is similar to the elephant test. Yet the primate test is commercially available, is considerably less expensive, and is regularly used by veterinarians in zoos and other institutions without federal oversight of specimen collection. We would like USDA to reconsider amending the licensing restrictions for the use of the Elephant Tb Stat-Pak® that require such federal supervision.
- Based on the discussion at the June 3 meeting, certification for accredited veterinarians to perform the Elephant Tb Stat-Pak® is being planned by APHIS Animal Care. We would like to have details on the protocol, criteria and timing for the certification. Specifically, will veterinarians need to be certified as laboratories in order to perform the tests?

- We would like USDA to outline the restrictions on the Elephant Tb Stat-Pak® test for use by certified veterinarians. Some earlier communications indicated that it would be available for use for species other than elephants and we would like clarification on the use of the tests for elephants as well as other species.
- Given the number of institutions and elephants that must be tested it is anticipated that individual federal oversight of all tests may be difficult to accomplish in a timely manner. If testing is not performed annually due to inability to schedule tests with Animal Care personnel, will an institution be held as non-compliant with the regulations?
- On a related note, the MAPIA is Chembio's proprietary test and is currently not readily available. At this time, Chembio must be contacted and asked if they are willing to run the test. It is our understanding that they are not setup to run the number of tests that may become mandated by these Guidelines. Furthermore, the cost of the test is at the sole discretion of Chembio and may be prohibitively expensive.
- We would like the USDA to outline the regulatory implications of the recommendations in the guidelines as they pertain to testing and movement of animals especially based on the Elephant Tb Stat-Pak® and MAPIA test status. The Guidelines suggest that an animal that is Elephant Tb Stat-Pak® positive be trunk washed more frequently (3 to 4 times a year). They also suggest that the sample be submitted to Chembio for MAPIA testing. They do not however address the status of an animal that is STAT-PAK positive but found to be negative for anti-bodies on MAPIA. How will such animals be classified for regulatory purposes?

Finally, we believe there are also areas of concern related directly to the Guidelines themselves that need to be addressed by the Tb Committee. A copy of our letter to the USAHA Tb Committee is attached here for your information, and a copy of this letter to you is also being copied to the Committee so they will have the full context of our concerns and discussions with USDA.
We appreciate the opportunity to address our concerns and look forward to your response.

(b)(6)

(b)(6)

cc:

AMERICAN ASSOCIATION OF ZOO VETERINARIANS
"Dedicated to wildlife health and conservation"
581705 White Oak Road • Yulee, Florida, 32097 • 904-225-3275 • Fax 904-225-3289

June 16, 2009

(b)(6) [redacted]

Tuberculosis Committee
United States Animal Health Association
522 Flora Vista Rd. NE
Olympia, WA 98506

Dear (b)(6) [redacted]

As you may know, since 1996 all elephants in USDA licensed facilities are tested at minimum annually for *Mycobacterium tuberculosis* (Mtb). Testing is done though culturing of respiratory samples collected via trunk wash and is conducted following the GUIDELINES FOR THE CONTROL OF TUBERCULOSIS IN ELEPHANTS established by the National Tuberculosis Working Group for Zoo and Wildlife Species.

Prior to 2008 the Working Group was an ad hoc advisory committee of veterinarians, researchers, and USDA employees, that periodically met (most recently in 2006) to review and revise the Guidelines. At the last meeting several important issues and proposed changes to the guidelines were debated and remained unresolved. In 2008 the Working Group was dissolved and was replaced by a subcommittee of the Tuberculosis Committee of the United States Animal Health Association (USAHA), consisting of a small subset of members from the Working Group. Revised Guidelines were issued by the Subcommittee in October 2008.

Under the 2008 Guidelines the collection of trunk was samples for bacterial culture continues to be the only recognized means of diagnosing elephants with Mtb. However, the revised Guidelines incorporated two new tests ancillary: an Elephant TB STAT-PAK® Assay and a Multi Antigen Print Immuno Assay (MAPIA). These tests are serological tests that detect Mtb antibodies in the blood using similar antigens, and reagents in a modified ELISA format. They are not intended to definitively identify infected animals but are simply screening tests that detect antibodies and may be helpful in identifying animals exposed to the Mtb bacteria. The revised Guidelines specify the use of these tests only as a complement to the trunk wash and to identify animals that should be trunk washed more frequently.

Both tests were developed by and are proprietary to Chembio, Inc, and are similar in design to existing tests for Tb in primates and humans. The company, in this particular instance, was issued a license by USDA for the elephant TB STAT-PAK test that restricted its sale and use to federal laboratories, namely the National Veterinary Services Laboratories (NVSL). At the time it was widely presumed that this merely meant that veterinarians would have to submit blood

samples to the NVSL in order to have the elephant TB STAT-PAK test run. In contrast, the secondary MAPIA test was not licensed and can only be used through Chembio itself.

In May, APHIS/Animal Care announced that it had adopted the Revised Guidelines along with certain new testing procedures that were not included in the Guidelines. Since that time, AAZV has worked with an ad hoc coalition of stakeholders (elephant organizations and exhibitors, elephant and zoo veterinarians, as well as scientific investigators, in an effort to address mutual concerns and questions about these new requirements.

Animal Care is proposing to implement the Revised Guidelines' recommendations regarding annual testing with the elephantTB STAT-PAK in two ways. First, by having the test conducted by a specially trained APHIS Animal Care veterinarian. Second, by permitting the test to be conducted by non-federal veterinarians who are licensed in the appropriate state, are accredited by USDA and are certified by Animal Care. However, in the case of the latter, an Animal Care veterinarian or inspector would need to be present to observe the test. We have a number of concerns and questions about these new requirements that are discussed in the enclosed copy of our letter to Acting Administrator of APHIS Kevin Shea.

During the course of our discussions with APHIS and Animal Care officials, it became apparent that there were also further concerns stemming from certain aspects of the Revised Guidelines, some of which had not been fully discussed with Working Group members prior to publication. The following questions and concerns are ones we believe should be addressed by the Committee.

1. The Revised Guidelines are not clear regarding the use of the STAT-PAK during the more frequent testing following a STAT-PAK positive sample. The Guidelines need clarification whether the STAT-PAK is to be performed at each trunk wash series (3-4 times a year) or just during the annual trunk wash series (1 time per year).

2. The Revised Guidelines suggest that an animal that is STAT-PAK positive be trunk washed more frequently (3 to 4 times a year). They also suggest that the sample be submitted to Chembio for MAPIA testing. They do not however address the status of an animal that is STAT-PAK positive but found to be negative for anti-bodies on MAPIA. Given that there is considerable data indicating that there are a significant number of these false positives, and that APHIS will rely upon the Revised Guidelines for regulatory purposes, the status of such animals should be specifically addressed in the Guidelines. The Committee specifically amended the language recommended by the Working Group to require submission of samples to Chembio for MAPIA testing.

3. Changes to the treatment guidelines for elephants are incorporated in the Revised Guidelines that fail to address the significant evidence demonstrating the toxicity of the drugs in elephants. The Revised Guidelines continue to rely on 15 year old treatment guidelines for humans that have been largely

abandoned by physicians due to severe side effects. The dosages and duration of treatments of elephants with drugs with severe side effects must be thoroughly reviewed by the Committee.

4. The process of MAPIA interpretation in not addressed in the Guidelines. Currently Chembio runs the (multiple antigen) antibody test and then based on proprietary criteria makes a determination of positive or negative for tuberculosis. The cut off levels used for each antigen, which antigens are selected for the test, and the final assessment are not published, poorly validated, and cannot be verified independently. To our knowledge they do not even provide a printed result of the antibody readings for documentation of the test, only a positive or negative final result.

As noted, because the Guidelines have been and will continue to be relied upon by APHIS a copy of this letter is being forwarded to APHIS along with our letter to Mr. Shea.

In light of the questions about the Revised Guidelines we respectfully request that the Committee reconvene a subcommittee to further review and revise the Guidelines. We further respectfully suggest that the members of that subcommittee equally include members who are directly involved in the care and treatment of elephants as well as regulatory officials, in order to ensure the effectiveness of the Guidelines.

We appreciate your attention and look forward to your response.

(b)(6)

Sincerely

(b)(6)

USDA Animal Care

United States
Department of
Agriculture

Animal and Plant
Health Inspection
Service

4700 River Road
Riverdale, MD
20737

APR 24 2009

(b)(6)
(b)(6) USAHA
PO Box 8805
3805 Oakland Avenue, Suite 101A
St. Joseph, MO 64508

Dear (b)(6)

The Animal and Plant Health Inspection Service (APHIS), Animal Care, is pleased to acknowledge the receipt of the USAHA Resolution 49, addressing revision of the elephant tuberculosis guidelines in use as a reference guide since 1998. USAHA has approved the 2008 revisions and made the resolution that APHIS adopt and implement the October 29, 2008, edition of the "Guidelines for the Control of Tuberculosis in Elephants."

APHIS is adopting the guidelines. An implementation plan has been developed and we will begin testing according to the recommended guidelines in July 2009 after Animal Care personnel are trained in the use of the new test.

We thank USAHA for their review and guidance in this matter and look forward to using these new tools to maintain the health and well-being of our US elephant population.

Sincerely,

for

Chester A. Gipson
Deputy Administrator
Animal Care

USDA Animal Care

APR 24 2009

United States Department of Agriculture

Animal and Plant Health Inspection Service

4700 River Road
Unit 80
Riverdale, MD
20737

301-734-7833

(b)(6)
(b)(6) United States Animal Health Association
PO Box 8805
3805 Oakland Avenue, Suite 101A
St. Joseph, MO 64508

Dear (b)(6)

The Animal and Plant Health Inspection Service (APHIS), Animal Care (AC), is pleased to acknowledge the receipt of the USAHA Resolution 40, regarding the soring of Tennessee Walking and other show horses. This resolution calls for the elimination of the abusive practice of soring and requests the United States Department of Agriculture (USDA). APHIS, AC, in cooperation with industry, to continue our vigilant monitoring of the Horse Protection Act (HPA) of 1970.

We agree that soring is one of the most significant welfare issues affecting any equine breed or discipline and appreciate the opportunity to provide you with more information about our enforcement efforts. As you mention, the American Association of Equine Practitioners (AAEP) issued a white paper on August 7, 2008 with recommendations on how the horse industry can work collaboratively to end the cruel practice of soring horses to accentuate their gaits. We believe the AAEP provided sound criticisms and suggestions, and we take all of its recommendations very seriously, particularly the recommendation to eliminate the Designated Qualified Persons (DQP) program.

The 1976 amendments to the HPA mandated that enforcement responsibility be shared among Federal and industry-certified inspectors, and they also imposed a $500,000 yearly appropriation limit on the enforcement of the HPA. To meet our mandated responsibilities, USDA began certifying horse industry organizations (HIOs) to hire DQPs. This arrangement extended our enforcement capabilities to cover more horse shows than could otherwise be included, given the aforementioned budget limitations. Any changes to USDA's current HPA enforcement arrangement would require Congressional action.

We believe this approach allows us to effectively enforce the HPA while maximizing the participation of the HIOs. Since 1999, our officials have worked with certified HIOs to develop Horse Protection Operating Plans. Signatory HIOs are required to consistently apply a uniform penalty structure to all HPA violators, even when APHIS officials are not in attendance. We also strive to ensure that DQPs and APHIS veterinary medical officers (VMOs) follow the same inspection standards. To this end, we conduct joint DQP-VMO training sessions where DQPs and VMOs train side-by-side in an intense program that incorporates both classroom elements and hands-on practice.

In addition, as recommended in the AAEP white paper, APHIS has worked to develop new technological tools for use in HPA enforcement to make the inspection process more uniform and objective. Two years ago, we announced the use of gas chromatography/mass spectrometry (GC/MS)—a technique that identifies the composition of chemical mixtures that are sometimes applied to horses' legs—as an HPA enforcement tool. GC/MS makes it easier to detect the presence of numbing substances and determine when horses have been subjected to chemical and/or mechanical means of soring. In 2008, APHIS introduced thermography—which employs the use of infrared cameras to detect heat and locate areas of inflammation—alongside our physical examinations. Thermography has been a useful tool to assist in the diagnosis of soring, and we have fully implemented its use within our inspection procedure for 2009.

We appreciate USAHA support and hope this information assures you of our commitment to enforcing the HPA. We will continue to work cooperatively with HIOs and other stakeholders to enhance public-private cooperation and maximize HPA enforcement. If you would like more information on USDA's HPA enforcement efforts, please visit the Horse Protection program's Web page at www.aphis.usda.gov/animal_welfare/hp/index.shtml.

Sincerely,

Chester A. Gipson

Chester A. Gipson
Deputy Administrator
Animal Care

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November 10, 2009

BY FACSIMILE

Dr. Chester A. Gipson
Deputy Administrator
Animal Plant and Health Inspection Services
U.S. Department of Agriculture
4700 River Road, Unit 97
Riverdale, MD 20737

Re: Outdoor Amusement Business Association

Dear Dr. Gipson:

I am writing to you today on behalf of the Outdoor Amusement Business Association (OABA) to express our outrage at recent comments attributed to APHIS personnel regarding the revised Guidelines for the Control of Tuberculosis in Elephants.

Representing over 10,000 predominately family-owned and small businesses in the mobile amusement industry, OABA members include many USDA-licensed exhibitors who participate in traveling circuses and zoos and exhibit or perform at county and state fairs and other community educational events. OABA remains actively engaged in ensuring that our members support responsible animal ownership and exhibition and we support USDA/APHIS in their efforts to enforce the Animal Welfare Act.

Recent events, however, have been brought to our attention that we believe require an explanation from APHIS. At the annual meeting of the American Association of Zoo Veterinarians this past month, APHIS employee Denise Sofranko was in attendance. During an open session that included an update on the soon to be released Guidelines for the Control of Tuberculosis in Elephants, several veterinarians questioned the new requirement that the test be administered only by a USDA veterinarian, given that most zoo vets administer similar tests on other species on a regular basis without this level of scrutiny or intervention.

KELLEY DRYE & WARREN LLP

Dr. Chester A. Gipson
November 10, 2009
Page Two

After several similar inquiries in the public forum, AAZV (b)(6) (b)(6) made the statement that "he would say what Ms. Sofranko could not" – **that the new requirement was the result of private owners and small exhibitors falsifying test results.** Ms. Sofranko was observed by several at the meeting to be shaking her head in agreement, though she did not comment publicly. Furthermore, several individuals attending the meeting confirmed that Ms. Sofranko had given that same explanation to them in private conversations.

Dr. Gipson, we take these accusations very seriously. If ANY licensed exhibitor or accredited veterinarian – regardless of their size or affiliation – is falsifying test results or tampering with field tests, we urge you to take appropriate action against those individuals immediately. However, to imply that an entire government policy is warranted because of the alleged – and to date unsubstantiated – actions of some unnamed individual or individuals – is unacceptable.

We look forward to your response. Thank you for your time and attention.

Sincerely,

(b)(6)

cc: (b)(6)
(b)(6) Outdoor Amusement Business Association (OABA)

Chembio Granted USDA License For Its ElephantTB STAT-PAK® Assay

Rapid Serological Screening for TB in Elephants

New York, December 1, 2007- (OTCBB: CEMI) – Chembio Diagnostic, Inc. (OTC BB: CEMI.OB – News) announces that it has been issued an United States Department of Agriculture (USDA) license for its ElephantTB STAT-PAK Assay designed to rapidly (< 20 minutes) detect tuberculosis (TB) in both Asian and African elephants. Chembio's other rapid serological TB assay – PrimaTB STAT-PAK received USDA licensure earlier this year. Both products belong to a larger family of rapid immunochromatographic assays currently under development at Chembio for the detection of TB in animals. Those include CamelidTB STAT-PAK, CervidTB STAT-PAK, and BovidTB STAT-PAK Assay kits that all employ Chembio's recently patented technology, (U.S. Patent Number 7,192,721 – Universal Rapid Test and Method for Detection of Tuberculosis in Multiple Host Species).

While TB has been recognized for over 2,000 years in elephant, it has recently been recognized as an important reemerging disease in captive elephants, particularly Asian elephants. *M. tuberculosis,* the most common cause TB classically, causes the same infection in humans as in elephants. Transmission of TB between elephant and human (zoonoses) has been documented.

TB remains a serious threat to captive elephants world-wide. Since 1994, TB has been diagnosed in over 12% of the US Asian elephant population. One-fourth (~15,000) of all the Asian elephants reside in captivity in 13 Asian countries. The high incidence of TB among humans in Asia suggests that many elephants within Asia may be also infected. Further information about TB in elephant is available from Elephant Care International (www.elephantcare.org).

Largely in response to the confirmed occurrence of TB in two captive elephants, Guidelines for the Control of Tuberculosis in Elephants were first developed and accepted as a policy by the USDA in 1998. These same Guidelines were again updated in 2000 and 2003. Testing elephant for TB is an essential component of elephant health care. The veterinary care of elephant is monitored by USDA under the Animal Welfare Act (AWA). The only available diagnostic test for TB in elephants, until the licensure of Chembio's ElephantTB STAT-PAK Assay, was mycobacterial culture of trunk wash specimens, a procedure similar to obtaining a sputum sample for TB in humans.

There is considerable evidence that this method has poor sensitivity and can only identify animals with extensive shedding of the organisms which typically occurs late in the disease. Recent published studies on Chembio's ElephantTB STAT-PAK Assay indicate that serology is able to detect TB in elephant as much as 4 years prior to culture of TB from trunk washes. The current 2007 proposed ElephantTB Guidelines, under review by the TB subcommittee of the United States Animal Health Association (USAHA), recommend incorporating Chembio's ElephantTB STAT-PAK Assay as an integral component in monitoring and managing TB in the US elephant population.

Chembio's ElephantTB STAT-PAK Assay is a lateral flow, onsite animal side test for the rapid detection of antibodies to TB in elephants. The test employs a unique and patented cocktail of recombinant proteins or antigens to both *M. tuberculosis* and *M. bovis*. The assay utilizes whole blood, serum, or plasma in very small aliquots of 30 µl to produce a qualitative result in less than 20 minutes.

USDA restricted the use of the assay in the US to only laboratories approved by state and federal animal health officials, of which currently the only one is NVSL (National Veterinary Services Laboratory) in Ames, Iowa. Therefore, Chembio's ElephantTB STAT-PAK Assay is currently commercially available for purchase only outside the US. Contact Chembio's website at www.chembio.com, e-mail your request to info@chembio.com, or contact Les Stutzman at LStutzman@chembio.com for more information.

ABOUT CHEMBIO

Chembio Diagnostics, Inc., a developer and manufacturer of rapid diagnostic tests for infectious diseases, is on the frontlines of the global battle against the AIDS pandemic. The Company has received marketing approval from the FDA for its SURE CHECK(R) HIV 1/2 and HIV 1/2 STAT-PAK(R) rapid tests, marketed in the United States by Inverness Medical Innovations. The Company also manufactures rapid tests for veterinary Tuberculosis and Chagas Disease. In March 2007 Chembio was issued a United States patent for the Dual Path Platform (DPP(TM)), a next generation lateral flow platform. DPP has demonstrated significant advantages over currently available lateral flow methods, including increased sensitivity, sample flexibility, and multiplexing capabilities.

Further information please visit www.chembio.com

FORWARD-LOOKING STATEMENTS

Statements contained herein that are not historical facts may be forward-looking statements within the meaning of the Securities Act of 1933, as amended. Forward-looking statements include statements regarding the intent, belief or current expectations of the Company and its management. Such statements are estimates only, as the Company has not completed the preparation of its financial statements for those periods, nor has its auditor completed the audit of those results. Actual revenue may differ

materially from those anticipated in this press release. Such statements reflect management's current views, are based on certain assumptions and involve risks and uncertainties. Actual results, events, or performance may differ materially from the above forward-looking statements due to a number of important factors, and will be dependent upon a variety of factors, including, but not limited to Chembio's ability to obtain additional financing, to obtain regulatory approvals in a timely manner, and the demand for Chembio's products. Chembio undertakes no obligation to publicly update these forward-looking statements to reflect events or circumstances that occur after the date hereof or to reflect any change in Chembio's expectations with regard to these forward-looking statements or the occurrence of unanticipated events. Factors that may impact Chembio's success are more fully disclosed in Chembio's most recent public filings with the U.S. Securities and Exchange Commission.

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CLINICAL AND VACCINE IMMUNOLOGY, July 2006, p. 722–732 Vol. 13, No. 7
1556-6811/06/$08.00+0 doi:10.1128/CVI.00133-06
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Tuberculosis in Elephants: Antibody Responses to Defined Antigens of *Mycobacterium tuberculosis*, Potential for Early Diagnosis, and Monitoring of Treatment

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Received 5 April 2006/Returned for modification 5 May 2006/Accepted 11 May 2006

Tuberculosis (TB) in elephants is a re-emerging zoonotic disease caused primarily by *Mycobacterium tuberculosis*. Current diagnosis relies on trunk wash culture, the only officially recognized test, which has serious limitations. Innovative and efficient diagnostic methods are urgently needed. Rapid identification of infected animals is a crucial prerequisite for more effective control of TB, as early diagnosis allows timely initiation of chemotherapy. Serology has diagnostic potential, although key antigens have not been identified and optimal immunoassay formats are not established. To characterize the humoral responses in elephant TB, we tested 143 serum samples collected from 15 elephants over time. These included 48 samples from five culture-confirmed TB cases, of which four were in Asian elephants infected with *M. tuberculosis* and one was in an African elephant with *Mycobacterium bovis*. Multiantigen print immunoassay (MAPIA) employing a panel of 12 defined antigens was used to identify serologic correlates of active disease. ESAT-6 was the immunodominant antigen recognized in elephant TB. Serum immunoglobulin G antibodies to ESAT-6 and other proteins were detected up to 3.5 years prior to culture of *M. tuberculosis* from trunk washes. Antibody levels to certain antigens gradually decreased in response to antitubercular therapy, suggesting the possibility of treatment monitoring. In addition to MAPIA, serum samples were evaluated with a recently developed rapid test (RT) based on lateral flow technology (ElephantTB STAT-PAK). Similarly to MAPIA, infected elephants were identified using the RT up to 4 years prior to positive culture. These findings demonstrate the potential for TB surveillance and treatment monitoring using the RT and MAPIA, respectively.

Tuberculosis (TB) caused by *Mycobacterium tuberculosis* or *Mycobacterium bovis* has recently re-emerged as an important infectious disease in exotic animals, particularly captive elephants (22, 24, 27). While TB has been recognized as a disease of elephants for over 20 centuries, increasing numbers of elephant TB cases have been reported in the past decade (13, 23). During 2001 to 2003, an outbreak of TB in a Swedish zoo involved five elephants and several other species (giraffes, rhinoceroses, and buffaloes), which were found to have been infected by four different strains of *M. tuberculosis* (13). In zoos and circuses, TB is more frequently detected in Asian elephants (*Elephas maximus*) than in African elephants (*Loxodonta africana*) (22, 27). Circumstantial evidence indicates that infection can be transmitted to elephants from humans and that it may spread between elephants and other susceptible species (13, 21, 23). Due to the zoonotic nature of the disease and close proximity of zoo/circus elephants to humans, the rise in elephant TB cases has potential public health ramifications (26), in addition to creating concern for the health and well-being of the animals.

The immunobiology of elephant TB is poorly understood. This lack of knowledge makes it difficult to develop and implement effective methods of testing, treatment, management, and control. In contrast to traditional (e.g., cattle) and nontraditional (e.g., various cervid species) livestock, infected elephants are often treated with antibiotics. Effective therapeutic regimens are more easily implemented if animals are diagnosed early in the course of infection. Early diagnosis also reduces the risk of exposure to other zoo animals, elephant handlers, and visitors to exhibits. Thus, it is critical to develop improved control strategies based upon early identification of infected animals.

After two Asian elephants were diagnosed with *M. tuberculosis* in 1996, the National Tuberculosis Working Group for

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TABLE 1. Diagnosis, treatment, and outcomes for culture-positive elephants[a]

Elephant	Age (yr),[b] date of diagnosis, and species	TB sign	MAPIA and RT (n)	Treatment	Status
L	46; October 1996, *M. tuberculosis*	Weight loss	Reactive (6)	INH/PZA/RIF over food for 12 mo; might be compromised by use of expired medications; restarted in 2001, but soon discontinued due to weakness	Relapse 3-4 yr after initial treatment; died of TB in February 2005, confirmed by positive culture and histopathology at necropsy
C	30; March 1997, *M. tuberculosis*	None	Reactive (12)	INH/PZA/RIF orally for 6 wk and then rectally for 17 mo	No positive trunk wash cultures after treatment; euthanized March 2004 for deteriorating health (degenerative joint disease); no evidence of TB at necropsy
K	31; May 2000, *M. tuberculosis*	None	Reactive (12)	INH/PZA rectally for 8 mo, followed by same plus RIF orally for 12 mo; multiple breaks due to intolerance; restarted in March 2003 with PZA; EMB; ENRO, AMK for 14 mo	Relapse and development of MDR strain after frequently interrupted treatment; euthanized June 2004 due to medication intolerance and risk of exposure to MDR TB; disease confirmed by culture and histopathology at necropsy
N	47; August 2000, *M. bovis* (postmortem)	None	Reactive (6)	None	Never trunk wash culture positive; euthanized August 2000 for chronic osteomyelitis; gross lesions and positive culture from tissues at necropsy
I	30; October 2000, *M. tuberculosis*	None	Reactive (12)	INH and PZA rectally for 12 mo	Cured (pending confirmation); monthly trunk washes negative for culture after treatment to date

[a] n, number of serum samples tested; RT, rapid test; MAPIA, multiantigen print immunoassay; INH, isoniazid; PZA, pyrazinamide; RIF, rifampin; EMB, ethambutol; ENRO, enrofloxacin; AMK, amikacin; MDR, multidrug resistant.
[b] At time of diagnosis.

Zoo and Wildlife Species was established to develop the best strategy of disease control for a variety of exotic animals. As a result, through the concerted effort of this group in conjunction with the United States Department of Agriculture (USDA), the *Guidelines for the Control of Tuberculosis in Elephants* were developed in 1997 (updated in 2000 and 2003). The guidelines include TB screening protocols and recommendations for antimicrobial therapy (25). The only USDA-recommended diagnostic test for TB in elephants is mycobacterial culture of trunk wash samples. However, there is a growing body of evidence indicating that this method of diagnosis has poor sensitivity, as it can only identify animals with extensive shedding of the organism that typically occurs late in the course of disease. Recent attempts to identify satisfactory alternatives for antemortem detection of TB in elephants using traditional techniques (i.e., tuberculin skin test) have been unsuccessful (13, 23). In contrast, antibody-based tests appear promising, but improved platforms and antigenic targets are needed (12, 31).

Multiantigen print immunoassay (MAPIA) was developed as an efficient tool for the identification of seroreactive antigens in human TB (17). More recently, this method was adopted to determine antigen recognition patterns in cattle, white-tailed deer, reindeer, and European badgers infected with *M. bovis* (9, 18, 33, 34). In the present study, we used MAPIA to characterize antigen recognition profiles and kinetics of immunoglobulin G (IgG) antibody responses to a panel of 12 defined antigens of *M. tuberculosis* and *M. bovis* in sera obtained from elephants with culture-confirmed TB. Serial serum samples collected before, during, and after treatment were tested. In addition, a rapid test (RT) for TB in elephants (ElephantTB STAT-PAK kit) was developed using lateral-flow technology and selected recombinant antigens. With the limited number of elephants tested, the RT showed the potential to be a simple and useful screening assay for early detection of TB in elephants, while MAPIA exhibited an added potential for monitoring of treatment and prediction of relapse.

MATERIALS AND METHODS

Animals and disease status. Five female elephants (L, C, K, N, and I) ranging from 30 to 47 years of age from different locations in the United States were diagnosed with TB by mycobacterial culture between 1997 and 2000. Table 1 summarizes epidemiologic, diagnostic, and treatment data for these animals. Two of them (L and C) had a history of exposure to *M. tuberculosis*-infected elephants. Four Asian elephants were culture positive for *M. tuberculosis* on the basis of trunk washes, and one African elephant (N) was culture positive for *M. bovis* at necropsy. The four Asian elephants were treated with antimycobacterial compounds for 12 to 20 months, whereas the African elephant did not receive therapy, as the diagnosis was made postmortem. Two of the treated animals, K and L, were given therapy repeatedly after the initial course, as they produced positive cultures of *M. tuberculosis* in posttreatment follow-up trunk wash testing. Disease was confirmed postmortem by culture and histopathology examination in the untreated African elephant infected with *M. bovis* and two of the four treated Asian elephants infected with *M. tuberculosis*. In one of the other two, which was euthanized for a severe joint disease, no evidence of TB was found 6 years after treatment. The second one remains alive and well at the time of this

writing. Additional clinical and diagnostic findings are presented in Results. Forty-eight serial serum samples (6 to 12 from each animal) collected from the five elephants before diagnosis as well as during and after treatment (the total observation period was 1.5 to 8.5 years) were used to measure the antibody responses by MAPIA and the RT. Ninety-five sera were also collected over time (for up to 10 years and 7 months) from 10 Asian females which had never been trunk wash culture positive, although they lived during 1986 to 2006 in the same five locations where the elephants with TB were identified. These samples were used as negative controls for the serological studies. Eight of the culture-negative elephants were exposed to the TB cases. All serum samples were stored frozen at −20°C or −70°C until testing.

Culture and drug susceptibility testing. Culture for *M. tuberculosis* and other mycobacteria was performed at the National Veterinary Services Laboratories (Ames, IA) in accordance with *Guidelines for the Control of Tuberculosis in Elephants* (25). The procedure for collecting triple trunk wash samples was described previously (23). In addition, specimens of feces, urine, vaginal discharge, and various tissues obtained at necropsy were processed for culture. The following solid media were used: Middlebrook 7H10 with glycerol, Middlebrook 7H11 with glycerol, Stonebrinks, and BBL Mycobactosel L-J; also, BACTEC 12B vials were inoculated with 0.5 ml of sample supplemented with PANTA (polymixin B, amphotericin B, nalidixic acid, trimethoprim, and azlocillin) and erythromycin (32 μg/ml). Processed specimens were inoculated on media and incubated at 37°C and 10% CO_2 for up to 8 weeks. All isolates and BACTEC bottles with a growth indicator value of >300 were confirmed with a GenProbe Accu-Probe *Mycobacterium tuberculosis* complex culture identification test. If the DNA probe test was positive, the niacin-nitrate reduction test was performed to confirm *M. tuberculosis* infection. Antimicrobial susceptibility testing was performed using BACTEC 460 and the following antibiotics: isoniazid (INH) at 0.1 μg/ml, rifampin (RIF) at 2 μg/ml, ethambutol (EMB) at 2.5 μg/ml, streptomycin (STR) at 2 μg/ml, and pyrazinamide (PZA) at 100 μg/ml.

Treatment. Culture-positive elephants were treated with first-line anti-TB drugs as recommended in *Guidelines for the Control of Tuberculosis in Elephants* in accordance with the protocol mentioned previously (23). Combinations of INH, RIF, and/or PZA were given daily or every 48 h, orally or rectally. Daily drug doses were 2.5 to 7.5 mg/kg for INH, 8 to 10 mg/kg for RIF, and 25 to 35 mg/kg for PZA. To treat multidrug-resistant (MDR) TB, a combination of PZA, EMB, enrofloxacin (ENRO), and amikacin (AMK) was used. Treatment duration varied from elephant to elephant, as indicated in Table 1.

Antigens. A panel of 12 defined proteins of *M. tuberculosis* was used in MAPIA, as described below. The following recombinant antigens were purified to near homogeneity as polyhistidine-tagged proteins: ESAT-6 and CFP-10 produced at the Statens Serum Institut (Copenhagen, Denmark); Mtb8 and Mtb48 supplied by Corixa Corp. (Seattle, WA); and MPB59, MPB64, MPB70, and MPB83 produced at the Veterinary Sciences Division (Stormont, United Kingdom). α-Crystallin (Acr1) and the 38-kDa protein were purchased from Standard Diagnostics (Seoul, South Korea). Native MPB83 protein was kindly supplied by Mark Chambers of the Veterinary Laboratories Agency (Weybridge, United Kingdom). Polyprotein fusion TBF10 was developed by Corixa Corp. and purified as described earlier (10). This construct included Mtb8, CFP10, and the 38-kDa protein. The hybrids CFP-10/ESAT-6 and Acr1/MPB83 were constructed by overlapping PCR using gene-specific oligonucleotides to amplify the genes from *M. tuberculosis* H37Rv chromosomal DNA. The fused polygene PCR products were cloned into the pMCT6 *Escherichia coli* expression vector using SmaI and BamHI restriction enzymes. The hybrids were purified to near homogeneity by exploiting the polyhistidine tag encoded by the vector.

MAPIA. MAPIA was performed as previously described (17). Briefly, purified antigens were immobilized on nitrocellulose membranes (Schleicher & Schuell, Keene, N.H.) at a protein concentration of 0.05 mg/ml using a semiautomatic microaerosolization device (Linomat IV, Camag Scientific Inc, Wilmington, DE) to generate invisible parallel bands. After antigen printing, the membrane was cut into strips 3.5 mm wide, perpendicular to the antigen bands, so that each strip carried all antigens. Next, strips were blocked for 1 h with 1% nonfat milk in phosphate-buffered saline containing 0.05% Tween 20 and then incubated with individual serum samples diluted 1:50 in blocking solution for 1 h at room temperature. After washing, strips were incubated for 1 h with peroxidase-conjugated protein G (Sigma) diluted 1:1,000 (Kirkegaard & Perry Laboratories), followed by another washing step. Elephant IgG antibodies bound to immobilized antigens were visualized with 3,3′,5,5′-tetramethyl benzidine (Kirkegaard & Perry Laboratories). MAPIA results were scored by two independent operators who did not know the true status of elephant samples, with a band of any intensity being read as a positive reaction. Results were also evaluated semiquantitatively by scanning and densitometry using Scion Image software (version Beta 4.0.2).

RT. Chembio Diagnostic Systems, Inc. (Medford, NY), developed an RT for TB in elephants (ElephantTB STAT-PAK kit) using lateral-flow technology. The antibody detection test employs a unique cocktail of several selected *M. tuberculosis* and/or *M. bovis* antigens and a blue latex bead-based signal detection system. The ready-to-use disposable device consists of a plastic cassette containing a strip of nitrocellulose membrane impregnated with test antigen and laminated with several pads made of glass fiber and cellulose. The test requires 30 μl of serum sample (plasma or whole blood) and 3 drops of sample diluent buffer (included in the kit) that are added sequentially to the sample pad. As the diluted test sample migrates to the conjugate pad, the latex particles conjugated to antigen bind antibody, if present in the sample, thus creating a colored immune complex. Driven by capillary forces, the complex flows laterally across the nitrocellulose membrane impregnated with specific antigen and binds to the immobilized antigen, thus producing a blue band in the test area of the device. In the absence of specific antibody, no band develops in the test window. The liquid continues to migrate along the membrane, producing a similar blue band in the control area of the device, irrespective of the presence of specific antibody in the test sample, demonstrating that the test reagents are functioning properly. Results are read 20 min after addition of the sample and diluent buffer either visually or by using a portable optical reader. Any visible band in the test area, in addition to the control line, was considered an antibody-positive result, whereas no band was considered a negative result. Semiquantitative evaluation of test band intensity in this study was performed by an in-house-developed computer-assisted optical reader designed to measure reflectance at 624 nm.

RESULTS

Clinical and diagnostic findings, treatment and pathology. The disease status and medical histories of five culture-positive and ten culture-negative elephants are presented below and summarized in Tables 1 and 2.

Elephant L. A 46-year-old Asian female elephant resided in a herd that had a history of TB. In late 1996, elephant L was diagnosed with *M. tuberculosis* by positive trunk wash culture. At the time, she was noticed to have lost a significant amount of weight. Several elephant handlers had tested positive for TB exposure a few months before elephant L was diagnosed (21). Treatment with INH, RIF, and PZA given with food was initiated in December 1996 and continued for 12 months. Although trunk washes were culture negative during follow-up testing, the quality of treatment might have been compromised, as the amounts of anti-TB drug administered were insufficient, based on current recommendations. In the following years, elephant L remained "excessively thin" and became emaciated by 2001, when her trunk washes again produced positive culture results. She was treated repeatedly for a short period in the second half of 2001 before the intervention was discontinued due to her weakness and poor acceptance of medications. Although follow-up testing of monthly trunk washes or feces did not yield positive findings, elephant L died of TB in February 2005. Weakness, depressed appetite, and dyspnea were noted several days prior to her death. Postmortem examination revealed multiple gross lesions compatible with TB in the lungs and lymph nodes. *M. tuberculosis* was cultured from the lungs, lymph nodes, liver, and uterus. The isolates obtained from elephant L in 1996, 2001, and 2005 were identical, indicating disease reactivation rather than reinfection. Since it was believed that she had long-standing disease combined with an inadequate treatment history, the increased likelihood of MDR TB was considered; however, drug susceptibility testing of the *M. tuberculosis* isolates failed to confirm this suspicion.

Elephant C. A 30-year-old Asian female elephant was originally a performer in a traveling circus before spending her last

TABLE 2. Disease status of culture-negative elephants[a]

Elephant	Age (yr)[b] and source of TB (exposure)	Other diagnosis	MAPIA and RT (*n*)	Treatment	Status
B	30; unknown	Chronic wasting disease	NR (1)	None	Died May 2004; no evidence of TB at necropsy
D1	60; elephant L	Osteomyelitis	NR (7)	None	Annual trunk washes negative for culture to date
T1	31; elephant C	Foot disease	NR (20)	INH orally for 5 mo; discontinued May 1998 due to poor acceptance	Euthanized March 2005 for foot problems; no evidence of TB at necropsy
P	41; unknown	Chronic arthritis	NR (7)	Anti-inflammatory drugs	Euthanized November 1992 for chronic arthritis; *M. intracellulare* isolated from lungs; no evidence of TB at necropsy
S1	36; elephant K	Glomerulonephritis	NR (15)	Antibiotics, aspirin, nutritional supplements	Died February 2003; no evidence of TB at necropsy
T2	31; elephant K	None	NR (22)	None	Quarterly trunk washes negative for culture to date
S2	25; elephant N	None	NR (1)	None	Annual trunk washes negative for culture to date
A	30; elephant I	None	NR (8)	None	Semiannual trunk washes negative for culture to date
D2	16; elephant I	None	NR (6)	None	Semiannual trunk washes negative for culture to date
R	12; elephant I	None	NR (8)	None	Semiannual trunk washes negative for culture to date

[a] *n*, number of serum samples tested; RT, rapid test; MAPIA, multiantigen print immunoassay; INH, isoniazid; NR, nonreactive.
[b] At time of TB diagnosis in same zoo.

several years in zoos. Elephant C had no symptoms suggestive of TB when she tested positive for *M. tuberculosis* from trunk wash cultures collected in April 1997. The diagnosis occurred weeks after she was transported from another zoo where an elephant had just died of *Salmonella enterica* infection but necropsy revealed evidence of pulmonary TB (*M. tuberculosis* was isolated from a mesenteric lymph node) and where three elephant handlers reportedly tested positive for TB exposure. The *M. tuberculosis* isolates were susceptible to all five antibiotics in vitro. Treatment with INH, RIF, and PZA given over food was initiated in May 1997. The treatment was changed 3 months later to drug administration via rectal suppository and completed in December 1998. Follow-up trunk wash cultures remained negative from July 1997 (2 months after initiation of oral treatment). In March 2004, elephant C was euthanized due to severe joint disease. No evidence of active TB was found at necropsy.

Elephant K. A 31-year-old Asian female elephant lived in a zoo that had no history of TB. Screening elephants for mycobacterial diseases included triple trunk swabs in 1997, replaced by triple trunk washes starting in 1998. In May 2000, testing of mucous discharge from the trunk yielded positive results for acid-fast bacilli (AFB) and culture of *M. tuberculosis*. During the following weeks, *M. tuberculosis* was also isolated from vaginal discharge and feces. No symptoms suggestive of TB were observed. Daily rectal therapy with INH and PZA was immediately initiated. After 2 months, treatment was changed to 48-h medication intervals for another 9 months, in accordance with the 2000 *Guidelines for the Control of Tuberculosis in Elephants*. Treatment was then changed to oral administration, and RIF was added to the protocol. Therapy continued for another 12 months, but on multiple occasions it was discontinued for several days to a few weeks due to poor appetite and depression. Follow-up trunk wash and vaginal discharge samples repeatedly tested culture negative for 1 year after treatment, until *M. tuberculosis* was detected in vaginal discharge and urine samples in February 2003. The isolate was found to be an MDR strain (resistant to INH and RIF). Treatment with PZA, EMB, and ENRO orally and AMK intramuscularly was initiated and continued for another year, but again there were frequent interruptions because of drug intolerance causing illness, poor appetite, and depression. Elephant K was euthanized in June 2004 due to recurring intolerance to medication and risk of exposure of staff and animal collection to MDR TB. A postmortem examination confirmed active disease with moderate to severe, chronic, diffuse granulomatous lymphadenitis with mineralization and moderate to severe, chronic, multifocal and coalescing granulomatous pneumonia with mineralization and rare intralesional AFB. Granulomatous lesions were also found in the uterus and abdomen. *M. tuberculosis* was cultured from the lungs and lymph nodes.

Elephant N. A 46-year-old female African bush elephant (*Loxodonta africana knochenhaueri*) resided in a zoo that had a history of *M. bovis* infection in other species. Elephant N had no signs suggestive of TB but suffered from a chronic nonresponsive osteomyelitis and was euthanized in August 2000. Other medical concerns included recurrent episodes of ventral edema and hyperprolactinemia. Screening for mycobacterial diseases by triple trunk swabs in 1997 or by annual triple trunk washes starting in 1998 had produced negative results. At necropsy, in addition to chronic osteomyelitis, an extensive granulomatous pneumonia was found, and *M. bovis* was cultured from several tissue samples. The isolate was resistant to PZA,

which was expected for *M. bovis*. Also, *Mycobacterium avium* was isolated from the frontal sinus.

Elephant I. A 30-year-old female Asian elephant was transported from a circus to a zoo in August 1997. Trunk wash cultures were performed nine times in 1998 with no growth of *M. tuberculosis*. Elephant I did not have any clinical signs of disease. In late 2000, three separate trunk washes collected in October and November produced positive cultures of *M. tuberculosis*. Treatment with INH and PZA rectally was initiated in late December 2000. Six weeks later, a follow-up trunk wash culture yielded a positive result. This culture contained two *M. tuberculosis* isolates, both of which were different from the original isolate. Of the three isolates, two were pansensitive to antitubercular drugs, while the third (one of those found during treatment) was monoresistant to RIF. Administration of INH and PZA continued for 1 year until January 2002 without any further positive cultures. Monthly trunk washes have continued to be culture negative to date.

Culture-negative elephants. Ten elephants from the same herds where the five TB cases had been found were included in this study as a control (TB-negative) group (Table 2). All were Asian females with a history of multiple "no isolation" trunk wash cultures. The average age in this group was similar to that of the elephants with TB (31.2 and 36.8 years, respectively). Of the 10 control elephants, eight had been exposed to TB, five had other maladies (i.e., chronic wasting disease, osteomyelitis, glomerulonephritis, and chronic arthritis), and two received antibiotic treatment (Table 2). Four elephants were confirmed as negative for TB, as no evidence of TB was found at necropsy when they died or were euthanized for other reasons.

Antibody responses and antigen recognition in MAPIA. Humoral immune responses in five elephants with TB were characterized by retrospective serological testing of sequential samples collected over time using MAPIA (Fig. 1). In elephant K, the IgG antibody responses were first detected in November 1996, 42 months before the diagnosis was made by trunk wash culture in May 2000. The earliest antigen recognized was ESAT-6, either alone or as a fusion protein with CFP10. The magnitude of the antibody response increased dramatically over time and involved multiple antigens consistently recognized during 1997 to 2000. In addition to ESAT-6, seroreactive proteins were CFP10, the 38-kDa protein, Acr1, MPB83, Mtb48, and the Acr1/MPB83 fusion protein. After treatment was initiated in May 2000, levels of antibodies to certain antigens declined while others remained unchanged in the following years.

Asian elephants C, L, and I, which were infected with *M. tuberculosis*, and African elephant N, which was infected with *M. bovis*, produced strong antibody responses to several antigens at the time of culture-based diagnosis. Their sera showed similar patterns of antigen reactivity in MAPIA, including ESAT-6, CFP10, and fusions containing these molecules. An anti-ESAT-6 IgG response in elephant N was first detected in February 1999, 18 months before *M. bovis* was isolated from tissues at necropsy. In elephant I, the only prediagnosis sample collected in September 1998 (over 2 years prior to positive trunk wash culture) was already reactive in MAPIA, showing a similar magnitude of antibody response with an almost identical antigen recognition pattern to that found at the time of culture-based diagnosis. No samples taken prior to diagnosis by culture were available from elephants C and L to establish the time of seroconversion. As observed in elephant K, the antibody levels in elephants C, L, and I gradually declined for several months after initiation of treatment and remained low or undetectable for many years thereafter. No antibody was detected by MAPIA in any serum sample from the consistently culture-negative elephants (Table 2).

Antibody detection by the RT. Lateral-flow technology offers a very simple and rapid way to detect antibody of interest in a diagnostic sample. Qualitative "yes-or-no" read-outs can be obtained within 20 min by visual evaluation. Also, if necessary, the intensity of test bands can be measured by an optical reader. Figure 2 shows examples of RT images obtained with selected samples from three elephants (K, N, and I) collected before seroconversion (nonreactive) and at the time of culture-based diagnosis (reactive). Reader-generated data (Fig. 3) demonstrated that antibody detection by the RT was generally in agreement with MAPIA results (Fig. 1). The RT detected antibody as early during infection as it was measurable by MAPIA or even several months earlier in one TB case. In elephant K, the March 1996 sample was reactive by the RT but still nonreactive by MAPIA (presumably due to the ability of the RT to detect IgM in addition to IgG). Strong RT bands were observed with sera from all five culture-positive elephants (Fig. 2 and 3), with optical reflectance readouts ranging from 257 units for elephant N to 728 units for elephant I obtained at the time of diagnosis. No antibody was detected in any of the consistently culture-negative elephants (Table 2). Optical reader values for all 95 sera in this group were 0.

Seroconversion and shedding. Availability of prediagnosis samples from three of the infected elephants provided an opportunity to compare the timing of antibody response onset to that of culture-based diagnosis. In elephant K, serum collected in March 1996 was positive by the RT, over 4 years earlier than the diagnosis made by culture in May 2000 (Fig. 3). By MAPIA, an IgG antibody response to ESAT-6 was first detected in September 1996, or 3.5 years earlier than *M. tuberculosis* was isolated from trunk washes (Fig. 1). For elephant I, a high-level antibody response was detected by both MAPIA and the RT in a sample collected in September 1998 (no earlier sample was available), over 2 years before the culture-based diagnosis (Fig. 1 and 3). Sera from elephant N were reactive by the RT and MAPIA 1.5 years prior to *M. bovis* isolation at necropsy. The remaining two culture-positive elephants had IgG responses already well established by the time of diagnosis (no prediagnosis serum samples were available). Thus, our observations strongly suggest that antibody-based assays have the potential to identify infected elephants long before shedding stages of the disease.

Effect of antimycobacterial therapy on antibody levels. As mentioned above, humoral immune responses in all elephants with TB were robust and well established at the time of diagnosis. Antimycobacterial therapy was administered to four of the culture-positive elephants (L, C, K, and I). In most cases, the RT remained strongly reactive during and after treatment, showing little change over time (Fig. 3). In contrast, MAPIA demonstrated that the levels of antibody to certain antigens declined rather quickly down to baseline in response to treatment. Figures 1 and 4 show MAPIA strips and corresponding semiquantitative data obtained with selected antigens by scan-

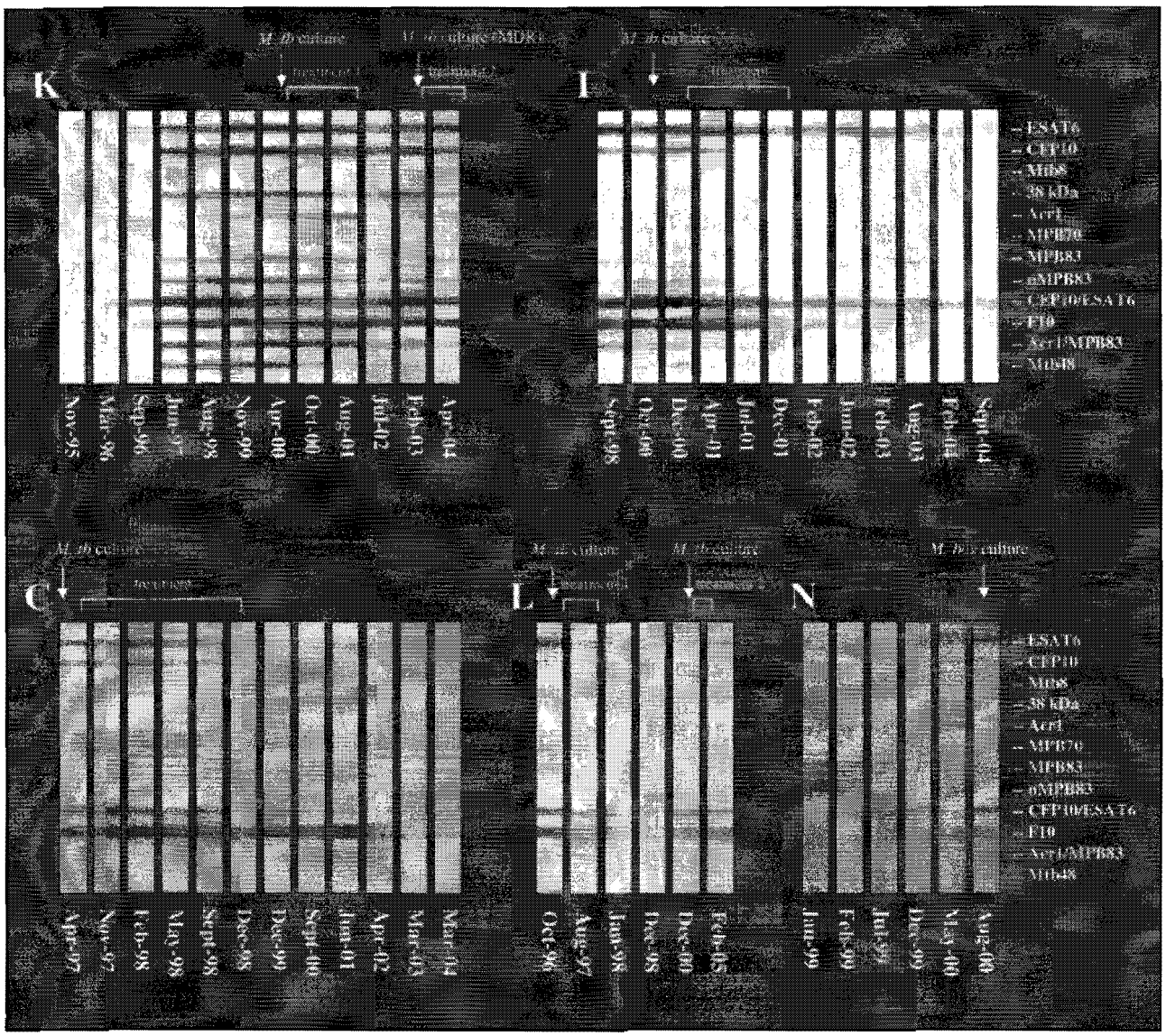
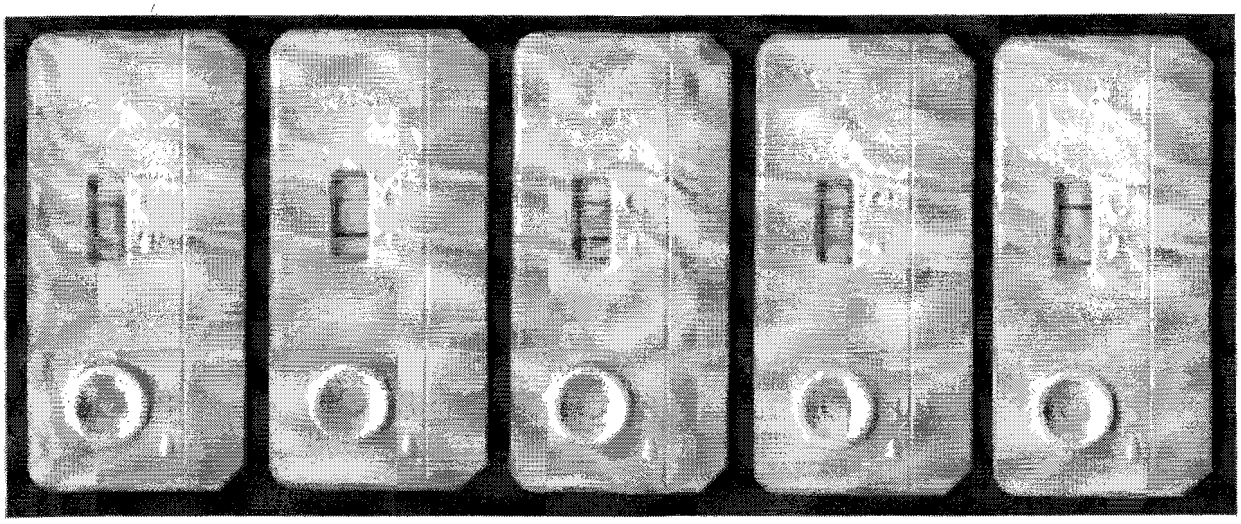


FIG. 1. Serum IgG antibody responses in culture-positive elephants to individual antigens of *M. tuberculosis*. MAPIA was performed as described in Materials and Methods. Each panel of strips represents a set of serum samples collected over time from one elephant, as shown (K, I, C, L, and N). Each strip represents one serum sample collected on the date indicated. Bands on MAPIA strips show the presence of antibodies to antigens printed (listed on the right). Time of diagnosis by positive culture is shown by arrows, and treatment duration is indicated for each elephant.

ning and densitometry. In elephant K, the antibody response to ESAT-6 (as well as a few other antigens) was not affected by therapy. In contrast, serum IgG reactivity to Acr1 and Mtb48, particularly, decreased to baseline levels during treatment. Interestingly, a short-lived spike of antibody response to Mtb48 in February 2003 appeared to coincide with a positive culture of *M. tuberculosis* from this elephant again, followed by its decline after treatment was reinitiated in March 2003. In elephants C and I, whose sera predominantly recognized ESAT-6 and CFP10, the antibody levels also decreased progressively and remained low or undetectable in the posttreatment period. In contrast, after a similar response to initial antimycobacterial therapy, elephant L showed a significant increase in antibody response in 2000, about 3 years after treatment and a year before another positive trunk wash culture. High levels of antibody lasted in this elephant until her death from TB in February 2005. Therefore, our findings demonstrate that MAPIA may have the potential for monitoring treatment and predicting relapses of TB in elephants.

DISCUSSION

Elephant TB has recently emerged as a serious infectious disease with zoonotic potential. Although it has been reported that about 3% of captive elephants in the United States are infected with *M. tuberculosis* (22, 27), recent estimates are up



1 2 3 4 5

FIG. 2. Rapid detection of serum antibodies by the ElephantTB STAT-PAK kit. The RT was performed as described in Materials and Methods. In the test window, the upper line is the control band and the lower line is the test band. Results were obtained with selected serum samples from elephant K (1, November 1995; 2, April 2000), elephant L (3, October 1996), and elephant N (4, August 2000; 5, January 1999). RT images 1 and 5 represent nonreactive samples collected from elephants K and N, respectively, before seroconversions. RT images 2 and 4 represent reactive samples from the same two elephants collected at the time of diagnosis by positive cultures of *M. tuberculosis* and *M. bovis*, respectively. Semiquantitative results obtained with an optical reader (intensity of test band measured as peak area) were as follows: 1, 0 units; 2, 714 units; 3, 458 units; 4, 257 units; and 5, 0 units.

to 6% (S. K. Mikota, unpublished data). Since the estimation is based on the results of trunk wash culture and this method has inadequate sensitivity, the true number of elephants harboring TB is likely higher. In humans, TB diagnosis is based on evaluation of clinical symptoms and laboratory methods, such as chest X-ray, smear microscopy, culture, or PCR probes (7, 29). In cattle, the intradermal skin test remains the primary screening tool in most countries, while the in vitro whole-blood gamma interferon Bovigam assay (Prionics AG, Schlieren, Switzerland) is generally used as an ancillary test (28). None of these methods provides reliable TB diagnosis in elephants. Trunk wash culture, the only officially recommended diagnostic test in the United States, has poor sensitivity. It is labor-intensive, time-consuming, and costly. This test requires three trunk wash samples collected on different days within 1 week. Both elephant and caretaker must be appropriately trained to obtain a trunk wash sample of acceptable quality. To further complicate a timely diagnosis, most elephants with active TB have no clinical signs of disease. In fact, four of the five culture-positive elephants described in the present study were asymptomatic at the time of shedding. Therefore, early diagnosis of TB in elephants can be very elusive.

The present study is the first attempt to define immunodominant antigens of *M. tuberculosis/M. bovis* in elephant TB. We found that ESAT-6 is strongly recognized by serum IgG in all culture-positive elephants. Less frequently reactive antigens are CFP10, MPB83, Mtb48, Acr1, and the 38-kDa protein. Polyepitope fusions containing one or two of these protein sequences performed especially well in MAPIA, consistent with our previous reports on human, cattle, and deer TB serology (10, 18, 33). The predominant recognition of ESAT-6 is also found in nonhuman primates infected with *M. tuberculosis* (4). However, in other host species, including humans, badgers, cattle, white-tailed deer, and reindeer, this antigen is not the most important antigenic target for serum antibodies (9, 15, 16, 18, 33, 34). Thus, it appears to be critical to determine the immunodominant antigens for each new species to be evaluated for TB serodiagnosis.

Humoral immunity in TB is characterized by individual variation in antigen recognition (15, 17). Both pathogen- and host-related factors are believed to determine this heterogeneity. It has been suggested that differential expression of stage-specific *M. tuberculosis* genes can lead to shifting profiles of the predominant immune recognition of corresponding proteins in the course of disease (5, 16). Latent TB infection in humans is reportedly associated with up-regulation of the gene encoding the 16-kDa heat shock protein known as α-crystallin (Acr1, Rv2031c), whereas other antigens may be predominantly recognized by T cells and/or antibodies in the acute phase of disease (5, 6). The present study, however, did not provide any evidence for significant changes in antibody profiles during elephant infection with *M. tuberculosis*, except in response to therapy.

Although antitubercular therapy in animals is not common, treatment is a preferred choice for culture-positive elephants in the United States. *Guidelines for the Control of Tuberculosis in Elephants* (25) defines general principles and protocols of treatment. Drug dosages and regimens are continuously optimized (19, 20, 35). Insufficient or improper treatment of TB in humans is the major cause of emerging MDR strains of *M. tuberculosis* (30). In elephant K, the initial isolate was susceptible to all first-line drugs but converted to an MDR strain when found again 1 year after a 20-month treatment involving multiple interruptions mostly due to drug intolerance. Further,

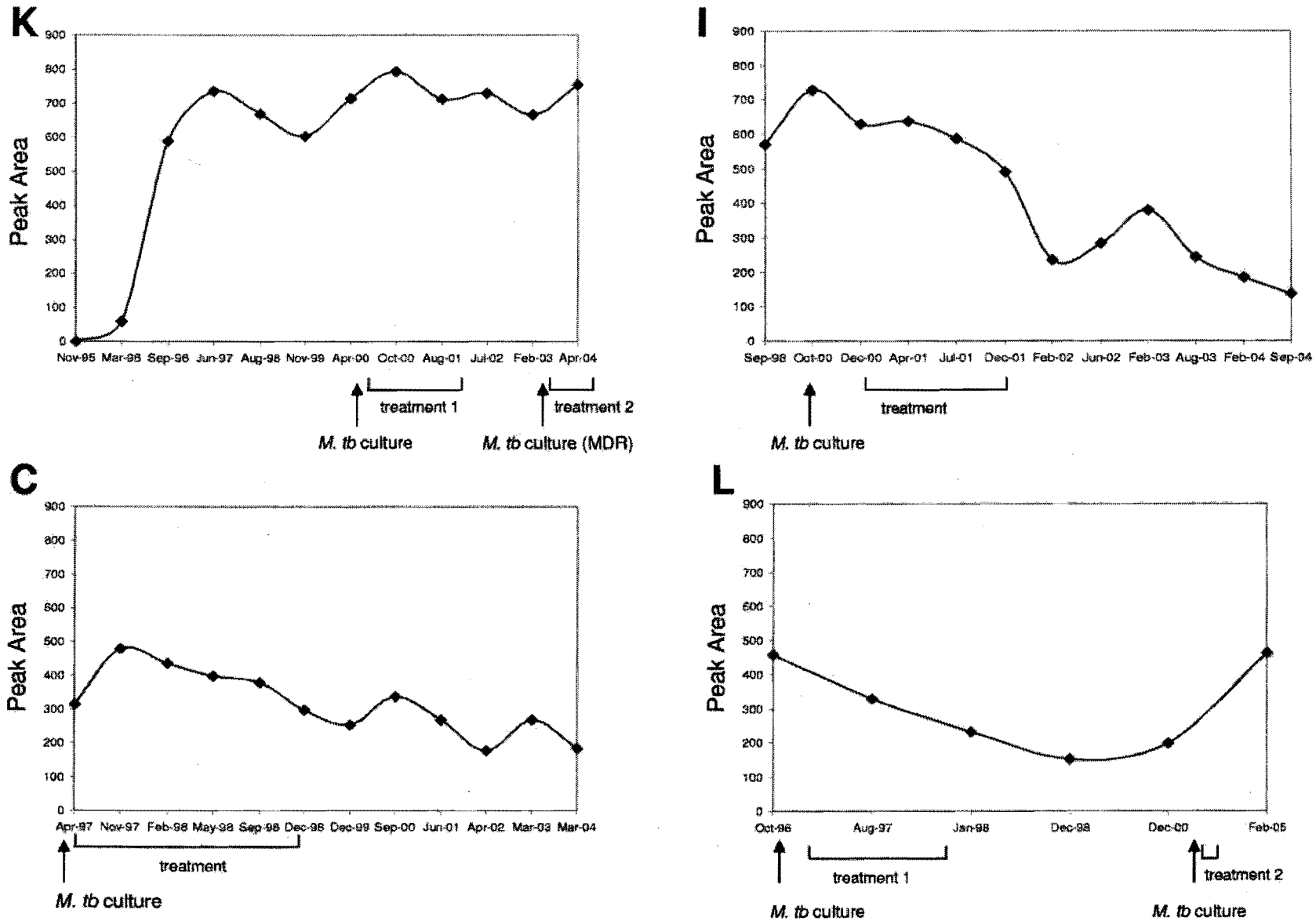


FIG. 3. Detection of serum antibodies by ElephantTB STAT-PAK kit in elephant TB before, during, and after treatment. The RT was performed as described in Materials and Methods. Results were measured with an optical reader and expressed in reflectance units (peak area). Each panel shows kinetics of antibody responses found by the RT with a set of serum samples collected over time from one elephant, as indicated (K, I, C, and L). The time of diagnosis by positive culture is shown by arrows, and treatment duration is indicated for each elephant.

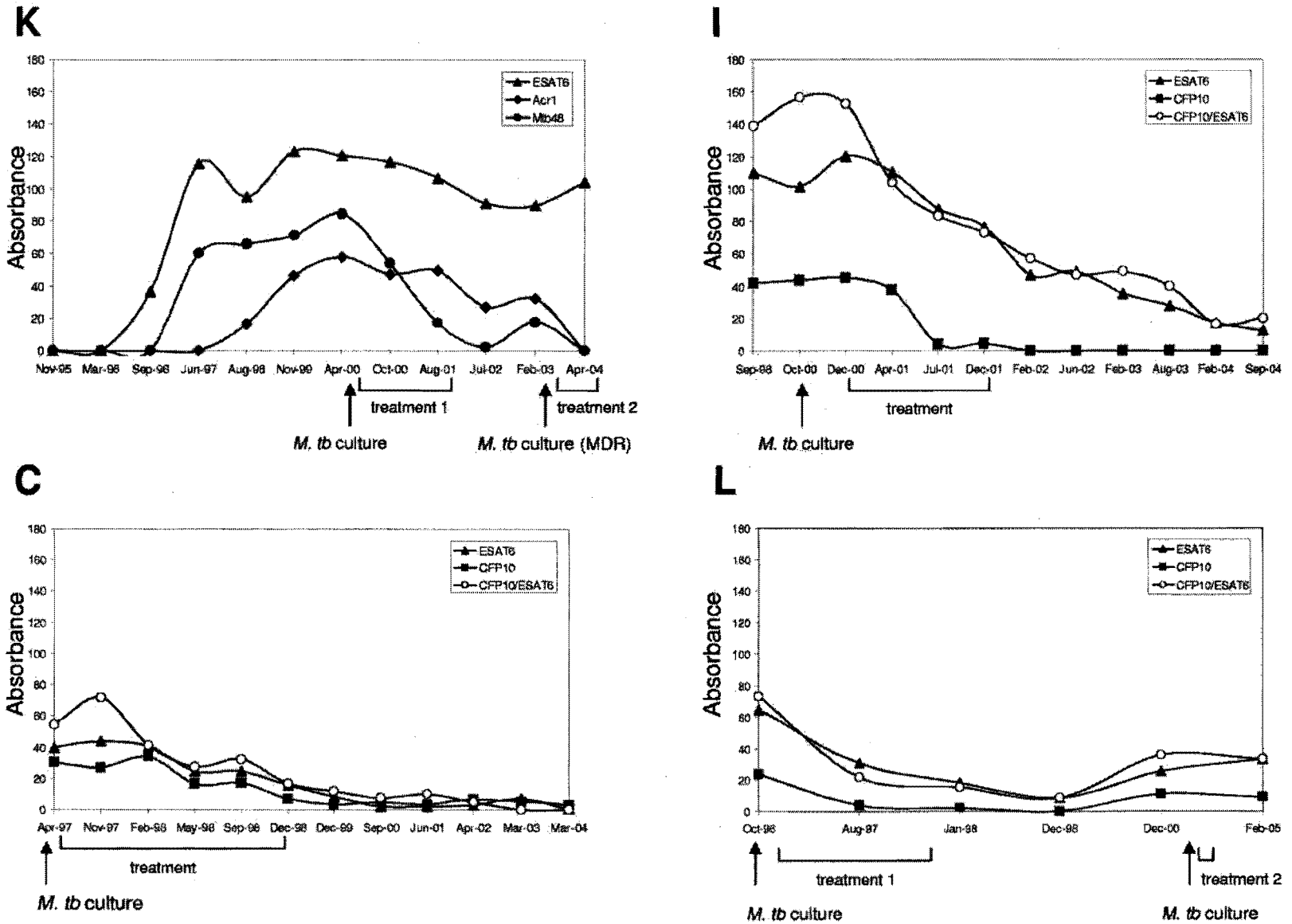


FIG. 4. Effect of antitubercular treatment on IgG antibody responses to selected antigens of *M. tuberculosis* in culture-positive elephants. MAPIA was performed as described in Materials and Methods. Each panel represents a set of serum samples collected over time from one elephant, as indicated (K, I, C, and L). Results are expressed in arbitrary units of optical density obtained from measuring intensities of MAPIA bands by scanning and densitometry using Scion Image (version Beta 4.0.2). The time of diagnosis by positive culture is shown by arrows, and treatment duration is indicated for each elephant.

in elephant I, three different isolates of *M. tuberculosis* were found, one of which was resistant to RIF but not to INH. This rare pattern of drug resistance is found mostly in TB patients with AIDS who receive highly active antiretroviral therapy in combination with antitubercular treatment including RIF (G. Simpson, personal communication).

The effects of antitubercular therapy on immune responses of human TB patients have been described (2). In most cases, antibody levels in seropositive patients declined within several months after start of treatment (3, 11). Monitoring of treatment is particularly important in situations where drug doses are not fully optimized and regimens are not well established. A recent elephant TB serology study showed that the antibody levels measured by enzyme-linked immunosorbent assay (ELISA) using *M. bovis* culture filtrate changed during treatment (12). In agreement with that observation, the MAPIA data presented here demonstrate that IgG to particular proteins of *M. tuberculosis* can be a sensitive marker for monitoring anti-TB treatment. Some antigens were more informative than others for predicting treatment outcomes, and they were not the same in all treated elephants. Therefore, the kinetics of antibody responses should be analyzed on a case-by-case basis for each antigen used in MAPIA.

As point-of-care diagnostics have demonstrated certain advantages, novel assay formats for infectious diseases developed in recent years become increasingly attractive. Therefore, the RT for detection of TB in elephants (ElephantTB STAT-PAK kit) was recently designed by Chembio Diagnostic Systems, Inc., and was used in the present study to evaluate its potential as a first-line TB screening tool. The RT is a disposable device that employs lateral-flow technology and a unique cocktail of recombinant antigens of *M. tuberculosis* and/or *M. bovis* to detect specific antibodies of three immunoglobulin classes, IgG, IgM, and IgA. The RT requires only 30 μl of serum, plasma, or whole-blood sample and provides results within 20 min. The RT is stable at room temperature for up to 18 months and requires no laboratory environment or skilled personnel, as it is easy to perform and read. If necessary, this assay can be supplemented by use of an optical reading instrument that would overcome the issue of subjectivity of visual evaluation and allow a higher degree of test standardization, generating quantitative results, establishing numerical cutoffs, and archiving data. In the present study, the RT showed the potential for early antibody detection in African and Asian elephants infected with *M. tuberculosis* or *M. bovis*. In one infected animal, the RT revealed seroconversion 6 months before MAPIA did, presumably due to IgM detection. Thus, the RT may be the most suitable screening tool to improve elephant TB diagnosis and surveillance.

In addition to the five TB cases, we tested 95 sera from ten culture-negative elephants (1 to 22 samples from each animal) collected between October 1986 and January 2006. No antibody was detected in any sample. Importantly, four of the ten elephants were confirmed negative controls, as they either died or were euthanized for various reasons and no evidence of TB was found at necropsy. In one of these elephants, *Mycobacterium intracellulare* was isolated from the lungs. Although further evaluation of the immunoassays will require testing greater numbers of elephants, the absence of false-positive results by MAPIA or the RT in the present study suggests high serodiagnostic specificity.

A multiantigen ELISA for elephant TB was described recently (12). In that study, three preparations of *M. bovis* (heat-inactivated culture filtrate, bovine purified protein derivative, and protein MPB70), two lipoarabinomannan antigens purified from virulent (Erdman) and avirulent (H37Ra) strains of *M. tuberculosis*, and avian purified protein derivative produced from *M. avium* were used. Among these antigens, the *M. bovis* culture filtrate showed the highest degree of serological discrimination between seven *M. tuberculosis* culture-positive and 40 negative elephants. The calculated sensitivity of the ELISA was 100%, and the specificity was 95%. The authors of that report recognized that "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". In the present study, we tested multiple serum samples collected long before positive culture results were obtained. In two elephants (K and N), for which earlier serum samples were available, retrospective testing revealed evident seroconversions. Most importantly, the antibody responses could be detected in infected elephants several years prior to shedding stages, demonstrating that serological assays might prove useful for early detection of TB in elephants. Antibody-based tests are unlikely to replace culture methods, as isolation of the pathogen from infected animals will always be important to confirm the diagnosis, identify the strain, and collect drug susceptibility data (23). However, management and control of TB in elephants and other nondomestic species would greatly benefit from early and rapid serodiagnosis. The cost of delayed diagnosis of TB in elephants may be extremely high. Elephants with undetected active TB may pose a serious threat of infection to other elephants, other animal species, and humans. Delayed diagnosis of TB in humans (>90 days) was found to increase rates of transmission to contacts (8) while placing the contacts at higher risk of infection. Therefore, earlier recognition of disease in elephants followed by immediate and appropriate interventions may prevent further spreading of *M. tuberculosis*.

Early stages of TB infection are associated with high expression of ESAT-6 (1), while the immune response to this antigen is known to correlate with progression and severity of disease (6, 32). In the present study, antibody to ESAT-6 was found in all culture-positive elephants by the time of diagnosis. Importantly, the response declined to baseline levels in those that appeared to be cured while remaining at high levels in those whose treatment turned out to be unsuccessful. The MAPIA predicted therapy failure in two elephants (L and K), as demonstrated by the sharp increase of antibody levels observed shortly before diseased animals started to shed organisms again. In human studies, higher rates of relapse after completion of therapy were found in TB patients who had prematurely suspended treatment (14). The other two treated elephants (C and I) produced MAPIA patterns predictive of cured TB without any relapse for several years after completion of therapy. One of these elephants was euthanized because of another disease and no evidence of TB was found at necropsy, whereas the other is alive and well at the time of this writing (over 4 years after treatment).

In summary, the present work improves our understanding of the immunobiology of elephant TB. We report here that

elephants diagnosed with *M. tuberculosis* produce robust antibody responses to multiple antigens long before positive cultures can be detected from trunk washes. These findings open the possibility of serological application for earlier detection of TB in elephants. We identified the key antigens of *M. tuberculosis* recognized by elephant IgG antibodies. We demonstrated the potential for the RT as an efficient TB screening tool and for MAPIA as a useful approach for treatment monitoring. Further, MAPIA utilizing antigens that are highly specific for the *M. tuberculosis* complex can constitute a confirmatory test, in addition to the RT, to avoid false-positive results due to potential cross-reactivity with non-TB mycobacteria. The use of such immunoassays will make it possible to identify infected elephants with high accuracy and start treatment at the earliest stages of disease, long before shedding, potentially leading to improved cure rates, decreased likelihood for development of MDR strains, and reduced transmission of infection.

ACKNOWLEDGMENTS

We are grateful to Mark Chambers for providing native MPB83 protein and to Raymond Houghton for providing recombinant Mtb8 and Mtb48 proteins.

This work was supported by Busch Gardens Tampa Bay and Chembio Diagnostic Systems, Inc.

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CLINICAL AND VACCINE IMMUNOLOGY, May 2009, p. 605–612 Vol. 16, No. 5
1556-6811/09/$08.00+0 doi:10.1128/CVI.00038-09
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Highly Accurate Antibody Assays for Early and Rapid Detection of Tuberculosis in African and Asian Elephants[▽]

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Received 27 January 2009/Returned for modification 23 February 2009/Accepted 26 February 2009

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.

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▽ Published ahead of print on 4 March 2009.

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TABLE 1. Study population

Group	Age range (yr)	No.				
		African	Asian	Male	Female	Total
Culture-confirmed TB	14–63	4	22	4	22	26
Exposed to TB	6–68	7	56	5	58	63
Healthy or other disease[a]	2–61	79	68	31	116	147
Total	2–68	90	146	40	196	236

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

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. tuberculosis* (n = 25) or *M. bovis* (n = 1). Of the 26 elephants, 7 died and 11 were humanely euthanized. TB was not necessarily the cause of death or the reason for euthanasia. Disease was diagnosed antemortem by trunk wash culture (n = 15; 58%) or only postmortem by isolating *M. tuberculosis* or *M. bovis* from various tissues (n = 11; 42%). Ten elephants were treated with first-line anti-TB drugs as recommended by the *Guidelines for the Control of Tuberculosis in Elephants* (33). The TB-exposed group included 63 elephants originating from 14 infected herds. Although no positive culture was ever isolated from trunk washes, some of these elephants could have been infected, as all of them had been in direct or indirect contact with known TB cases. A proportion of this group may have received prophylactic treatment, but the exact number of treated animals remains unknown due to nondisclosure policies for treatment regimens. The noninfected group consisted of 147 elephants without clinical symptoms suggestive of TB, with consistently negative trunk wash culture results, and with no history of potential TB exposure in the past 5 years. This group included 134 healthy animals and 13 with alternative diagnoses, such as chronic wasting syndrome, osteomyelitis, glomerulonephritis, chronic arthritis, or mycobacteriosis other than TB (MOTT). The last subgroup of four elephants included lung infection with *Mycobacterium intracellulare*, lymph node infection with an unidentified atypical mycobacterium, and two cases of fatal mycobacteriosis caused by *Mycobacterium szulgai* (9). Eighteen elephants were confirmed TB negative postmortem, as they died or were euthanized for other reasons and no evidence of TB was found at necropsy. Banked sera and freshly collected blood specimens were used for both retrospective and prospective serologic studies. All serum samples were stored frozen at −20°C or −70°C until they were tested.

Culture. The procedure for collecting triple trunk wash samples for culture was performed as described previously (25). Feces, urine, vaginal discharge, and various other tissues obtained at necropsy were also submitted for culture testing. Isolation and identification of *M. tuberculosis* and other mycobacteria were performed at the National Veterinary Services Laboratories (Ames, IA) and other certified laboratories, in accordance with the *Guidelines for the Control of Tuberculosis in Elephants* (33). Briefly, Middlebrook 7H10 with glycerol, Middlebrook 7H11 with glycerol, Stonebrinks, and BBL Mycobactosel L-J media, as well as BACTEC 12B vials, were inoculated with 0.5 ml of sample supplemented with PANTA (Becton Dickinson) and erythromycin (32 μg/ml). Processed specimens were inoculated on media and incubated at 37°C and 10% CO_2 for up to 8 weeks. All isolates and Bactec bottles with a growth indicator value of >300 were confirmed with the AccuProbe *Mycobacterium tuberculosis* Complex Culture Identification Test (Gen-Probe, San Diego, CA). If the DNA probe was positive, the niacin-nitrate reduction test was performed to confirm *M. tuberculosis*. MOTT species were identified by Gen-Probe AccuProbe Culture Identification Tests, according to the manufacturer's recommended procedures.

Electrophoresis and immunoblot assay. The antibody responses of the elephants were evaluated over time by immunoblot analysis using procedures described previously (6). Briefly, 115 μg of a whole-cell sonicate (WCS) of *M. bovis* strain 95-1315 was electrophoresed through preparative 12% (wt/vol) polyacrylamide gels. Electrophoretic transfer of proteins onto pure nitrocellulose was accomplished with the Bio-Rad Trans Blot Cell (Bio-Rad Laboratories, Mississauga, Ontario, Canada) using sodium phosphate buffer (25 mM; pH 7.8) at 0.8 Å for 90 min. After transfer, the filters were blocked with phosphate-buffered saline (PBS) containing 0.05% Tween 20 (Sigma) and 2% (wt/vol) bovine serum albumin (PBST-BSA). After being blocked, the filters were placed into a 20-slot miniprotean II multiscreen device (Bio-Rad), and individual sera (diluted 1:200 in PBST-BSA) were added to independent slots. After 2 h of incubation at 24°C with gentle rocking, the blots were washed three times with PBST and incubated with peroxidase-conjugated protein L (Sigma) diluted 1:2,500 in PBST-BSA for 1.5 h. The blots were again washed three times with PBST 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 Laboratories). The following recombinant antigens were immobilized on nitrocellulose membranes: ESAT-6 and CFP10 proteins, as well as the hybrids CFP10/ESAT-6 and Acr1/MPB83 produced at the Statens Serum Institut (Copenhagen, Denmark); MPB59, MPB64, MPB70, and MPB83 produced at the Veterinary Sciences Division (Stormont, United Kingdom); alpha-crystallin (Acr1) and the 38-kDa protein purchased from Standard Diagnostics (Seoul, South Korea); native MPB83 protein supplied by the Veterinary Laboratories Agency (Weybridge, United Kingdom); and Mtb8 and Mtb48 proteins and polyepitope fusion TBF10 developed by Corixa Corp. (Seattle, WA), as described previously (8). MAPIA results were scored by two independent operators who did not know the true infection status of the elephants, with a band of any intensity being read as a positive reaction.

ElephantTB Stat-Pak assay. The one-step lateral-flow test ElephantTB Stat-Pak (Chembio Diagnostic Systems, Inc., Medford, NY) employed selected *M. tuberculosis* antigens and a blue latex signal detection system for rapid detection of antibodies, as previously described (16). The test required 30 μl of elephant serum or plasma and 3 drops of sample buffer (included in the kit), which were added to the device sequentially. The results were read visually 20 min later by two independent operators who did not know the true infection status of the elephants. Any visible band in the test area, in addition to the control line, was considered an antibody-positive result, whereas no test band was considered a negative result.

DPP VetTB assay. A new-generation point-of-care test for TB in elephants was developed using Chembio innovative DPP technology. Unlike the ElephantTB Stat-Pak kit, which employed a conventional lateral-flow method, the DPP VetTB assay has two nitrocellulose strips that are connected in a "T" shape inside the device to allow independent delivery of test sample and antibody-detecting reagent. The first strip receives a serum sample and buffer solution via the sample well. The diluted sample migrates toward the second strip, containing two test lines (MPB83 and CFP10/ESAT-6 printed as separate bands) and one control line. Adding buffer to the conjugate well releases dried colloidal gold particles coupled with protein A/G and facilitates its migration along the second strip to the test area. If antibody is present in the sample, it binds to the immobilized test antigen, and the gold particles then react with this immune complex, thus making the test band visible. In the absence of detectable antibody, no specific immune complex would be formed on the test line, and therefore, no visible band would appear in the test area. The control band would develop, as the gold particles continue migrating along the second strip irrespective of the presence of antibody, ensuring correct performance of the test. The DPP VetTB assay was performed using 5 μl of elephant serum, 2 drops of buffer in the sample well, and 4 drops of buffer in the conjugate well. The results were read at 15 min visually by two independent operators who did not know the true infection status of the elephants or by using a DPP optical-reader device measuring reflectance in relative light units (RLU). Reactivity of CFP10/ESAT-6 and/or MPB83 above the cutoff value of 3.0 RLU was considered a positive result for the presence of antibody. No measurable reactivity with either of the two test antigens was taken as an antibody-negative result.

DBS. BloodStain cards (Whatman, Inc., Florham Park, NJ) were used according to the manufacturer's protocol to make dried blood spots (DBS) with elephant whole blood, serum, and plasma. Two antibody-positive and two negative control samples of each type from the same group of elephants were applied in duplicate to BloodStain cards (100 μl within each circle) and dried in the open air for 2 h at room temperature, and then the cards were sealed in plastic bags with desiccant and stored at +4°C. One and 5 weeks later, samples were extracted from the DBS by incubating each circle, cut into several pieces, in 200 μl of PBS overnight at +4°C. The eluted specimens were tested by serologic assays. For reference samples, aliquots of the positive and negative sera used for making DBS were kept frozen at −20°C until they were tested.

Data analysis. The TB-infected and noninfected groups were used for evaluation of the diagnostic sensitivity and specificity of the serologic assays. The TB-exposed group was considered only for seroprevalence analysis. The diagnostic performance of the serologic assays was evaluated against the gold stan-

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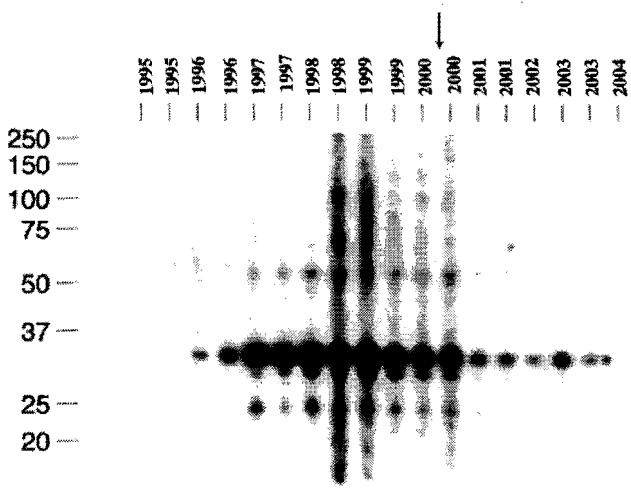

FIG. 1. Immunoblot to *M. bovis* WCS. Archived serum samples from an elephant infected with *M. tuberculosis* were evaluated for reactivity to *M. bovis* WCS by standard immunoblotting techniques. The samples are arranged sequentially, with the serum collection date (year) indicated at the top and molecular mass (Kilodaltons) on the left. The arrow indicates the time when the culture of *M. tuberculosis* was isolated from trunk wash samples and treatment was initiated.

dard of *M. tuberculosis* or *M. bovis* culture using available software (http://faculty.vassar.edu/lowry/VassarStats.html). The test sensitivity and specificity, as well as disease prevalence, were calculated for each assay and are presented with the 95% confidence interval (CI).

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, ~32kDa, 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 (i.e., 2% of the noninfected group), but 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.

TABLE 2. Seroreactivity rates of *M. tuberculosis* proteins in MAPIA with serum samples from culture-confirmed and control elephants

Antigen	No. (%) in group:	
	TB (n = 26)	Control (n = 147)
ESAT-6	24 (92)	0 (0)
CFP10	21 (81)	0 (0)
MPB83	15 (58)	3[a] (2)
Mtb48	5 (19)	0 (0)
MPT70	3 (12)	0 (0)
Acr1	2 (8)	0 (0)
Mtb8	1 (4)	0 (0)
38 kDa	1 (4)	0 (0)
CFP10/ESAT-6	26 (100)	0 (0)

[a] In all three cases, antibodies to MPB83 protein were found in elephants with MOTT.

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;

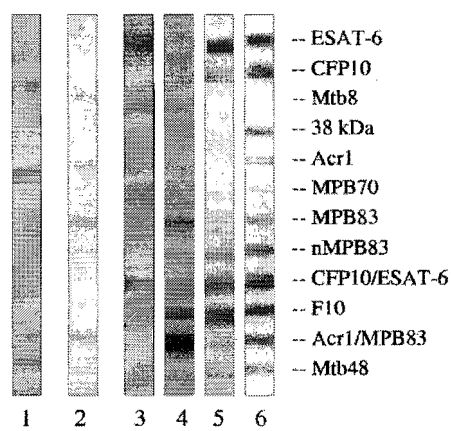

FIG. 2. Differential antigen reactivity by serum IgG obtained from *M. tuberculosis*-positive elephants. The images represent MAPIA strips developed with sera from a noninfected elephant (sample 1), an elephant infected with *M. szulgai* (sample 2), and four elephants infected with *M. tuberculosis* (samples 3 to 6). The printed antigens are listed on the right.

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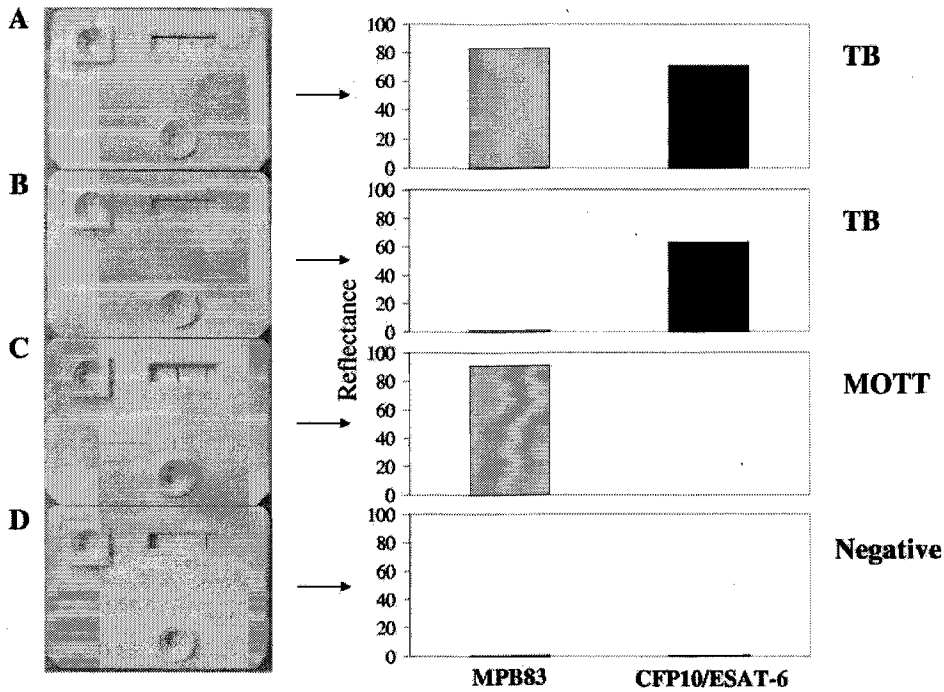

FIG. 3. Antibody detection by DPP VetTB assay. The images represent examples of typical results obtained for elephant TB (A and B), MOTT due to *M. szulgai* (C), or noninfected control (D). Reflectance values in RLU generated by a DPP reader device for the MPB83 test band (gray bars) and the CFP10/ESAT-6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

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

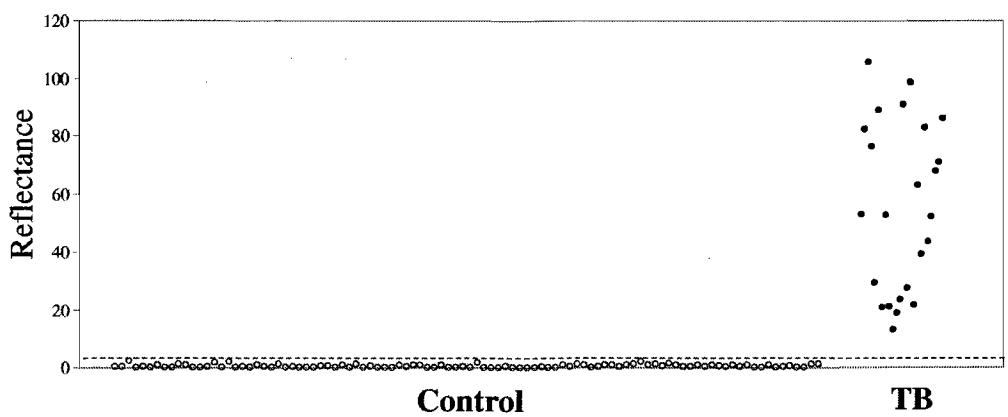

FIG. 4. Quantitative detection of serum IgG antibodies by DPP VetTB in culture-confirmed elephants. Reflectance values in RLU generated by a DPP reader with a CFP10/ESAT-6 test band are shown for sera from the 26 elephants with TB (solid circles) and for 100 randomly selected noninfected controls (open circles). A cutoff value of 3.0 RLU (dotted line) was established with the control sera as the mean plus 5 standard deviations.

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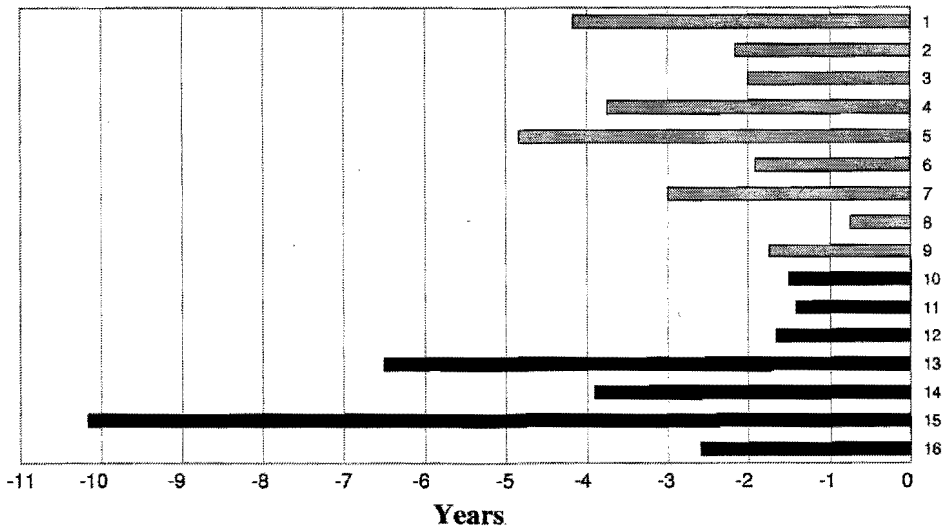

FIG. 5. Seroconversion time to first positive culture. Individual times are shown in years between initial MAPIA-positive samples collected from each elephant with TB and the first positive cultures of *M. tuberculosis* or *M. bovis* isolated from trunk wash samples (gray bars) or at postmortem examination (black bars).

mycobacteria. Two to seven different mycobacterial species were collected from each elephant, including *Mycobacterium avium* complex ($n = 5$), *M. intracellulare* ($n = 2$), *Mycobacterium gordonae* ($n = 2$), *Mycobacterium fortuitum* ($n = 2$), *Mycobacterium abscessus*, *Mycobacterium asiaticum*, *Mycobacterium chelonae*, *Mycobacterium flavescens*, *Mycobacterium mucogenicum*, *Mycobacterium nonchromogenicum*, *Mycobacterium simiae*, and *Mycobacterium 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, sera from 22 animals were reactive by the ElephantTB Stat-Pak kit (35%), 10 of which were also positive for CFP10/ESAT-6 antibody by MAPIA or the DPP VetTB test (16%). The discrepancies between the assays might be partially due to ElephantTB Stat-Pak false-positive reactions, to the ability of the technique to detect antibodies of all immunoglobulin classes (thus proving potentially more sensitive), or to the fact that some of the exposed elephants had been subjected to antimycobacterial therapy that may have prevented shedding and reduced the antibody responses to select antigens. Anti-TB treatment is known to affect the performance of the serologic assays over time differently (16), so that a treated elephant could remain reactive by ElephantTB Stat-Pak but eventually become nonreactive by MAPIA or the DPP VetTB test.

Seroprevalence of TB in elephants. With the close correlation found between the presence of circulating CFP10/ESAT-6-reactive antibody and TB in elephants, we estimated the prevalence of disease in the study population by using either culture or serology data (Table 3). While the overall estimates were 11.0% and 15.3% by culture or antibody detection, respectively, a fivefold-higher rate of seropositivity (21.9%, a statistically significant increase) was found among Asian elephants than among African elephants. Unlike the Asian elephant population, African elephants had no difference be-

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

Elephant species	Prevalence [% (95% CI)]	
	Culture[a]	ESAT-6/CFP10 antibody[b]
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)

[a] Trunk wash and/or postmortem testing.
[b] MAPIA and DPP VetTB data.

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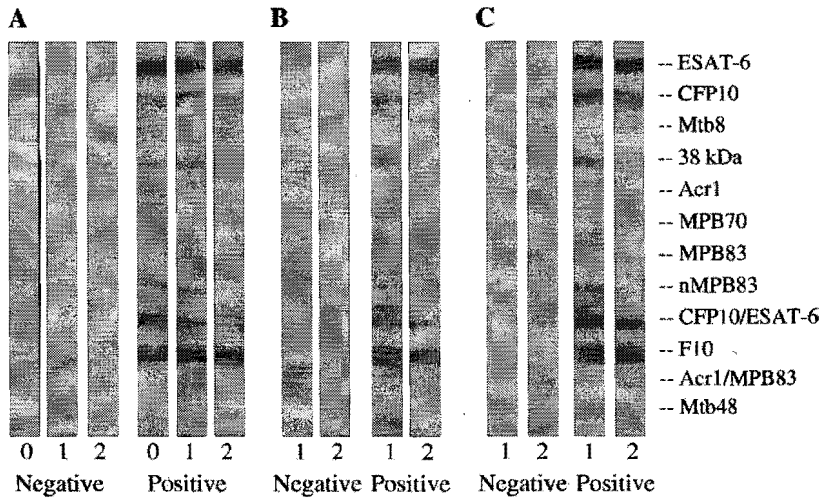

FIG. 6. MAPIA testing of DBS samples. The images represent MAPIA strips developed with serum (A), plasma (B), or whole-blood (C) samples eluted from DBS after 1 week (1) or 5 weeks (2) of storage, in comparison with the standard positive and negative sera used for making DBS (0). The printed antigens are listed on the right.

tween 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

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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. 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. tuberculosis*. The predominant serologic recognition of ESAT-6 has been reported for nonhuman primates infected with *M. tuberculosis* or *M. 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. szulgai* (9) or in cattle experimentally inoculated with *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. 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. tuberculosis* or *M. 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.

ACKNOWLEDGMENTS

We are grateful to Peter Andersen, Mark Chambers, Raymond Houghton, Jim McNair, and John Pollock for providing purified antigens, as well as to the numerous zoo veterinarians and elephant caretakers for supplying serum samples.

This work was supported by Busch Gardens Tampa Bay and Chembio Diagnostic Systems, Inc.

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