GUIDELINES FOR THE CONTROL OF TUBERCULOSIS IN ELEPHANTS 2010

UNITED STATES ANIMAL HEALTH ASSOCIATION (USAHA) ELEPHANT TUBERCULOSIS SUBCOMMITTEE

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These guidelines are available on the Internet at the following sites:

1. http://www.aphis.usda.gov/animal_welfare/index.shtml (available to the public)
2. www.aazv.org (available to AAZV members by password)
3. www.elephantcare.org (available to the public)
4. www.elephanttag.org (available to the public)

1. INTRODUCTION

Tuberculosis (TB) is caused by bacteria in the genus *Mycobacterium*. Over 100 species comprise this genus. Mycobacteria infect a broad range of species including humans, non-human primates, carnivores; marine mammals, psittacine birds, reptiles, fish, artiodactylids, pachyderms, and domestic and non-domestic ungulates. Species susceptibility to specific mycobacteria varies (Montali 2001).

In mammals, the term "tuberculosis" is used to define disease caused by *Mycobacterium tuberculosis* (*M. tb*) complex organisms. The *M. tb* complex includes *M. tuberculosis, M. bovis*, *M. africanum*, *M. microti, M. canetti, M. caprae, and M. pinnipedii*. A vaccine strain derived from *M. bovis* (*M. bovis BCG*) is sometimes included as a separate member of this complex.

The term "mycobacteriosis" refers to infection with any mycobacteria but is generally used to define disease caused by non-tuberculous mycobacteria (NTM). "Atypical mycobacteria" or "mycobacteria other than TB" (MOTT) are other terms used to describe this group. Most NTM are saprophytes found in soil or water but they may occasionally cause disease in humans and animals, including elephants.

Mycobacterium tuberculosis is the predominant disease-causing agent in elephants although cases caused by *M. bovis* have occurred. *Mycobacterium szulgai*, an uncommon NTM species, was associated with fatal disease in two African elephants (Lacasse 2007) and *Mycobacterium elephantis*, a rapidly growing mycobacterium, was isolated from a lung abscess of an elephant that died of chronic respiratory disease (Shojaei 2000). *Mycobacterium avium* is commonly isolated from elephants (Payeur 2002), but to date has not been associated with clinical disease.

The National Tuberculosis Working Group for Zoo and Wildlife Species has been monitoring TB in elephants since 1996. The original Guidelines for the Control of Tuberculosis in Elephants were released in 1997 and modified in 2000, 2003, and 2008. The Guidelines include recommendations for the testing, treatment, and surveillance of TB in elephants and are revised as new information becomes available. The 2010 guidelines include updated information on diagnostic tests and add further clarification to TB management groups.

2. DEFINITIONS

Ancillary diagnostic test: A subordinate or auxiliary test to be used in support of a primary test to diagnose disease.

Airborne transmission. Airborne transmission occurs by dissemination of either airborne droplet nuclei or small particles in the respirable size range containing infectious agents that remain infective over time and distance (e.g., spores of *Aspergillus* spp, *Mycobacterium tuberculosis* bacilli). Microorganisms carried in this manner may be dispersed over long distances by air currents and may be inhaled by susceptible individuals who have not had face-to-face contact with (or been in close proximity to) the infectious animal or person (Siegel 2007).

Attending veterinarian: a person who has graduated from a veterinary school accredited by the American Veterinary Medical Association's Council on Education, or has a certificate issued by the American Veterinary Medical Association's Council on Education Commission for Foreign Veterinary Graduates; has received training and/or experience in the care and management of the species being attended; and who has direct or delegated authority for activities involving animals at a facility subject to the jurisdiction of the Secretary (i.e. a USDA licensed facility).

Atypical mycobacteria: see non-tuberculous mycobacteria

Contact transmission:

Direct contact transmission may occur during activities such as touching or riding an elephant, being touched by an elephant, examining, medicating, bathing, and handling

Indirect contact transmission involves contact with a contaminated intermediate object, such as occurs during cleaning cages and equipment and handling soiled laundry. Injuries from contaminated sharps, such as scalpel blades, needles, and necropsy knives, may result in exposure to pathogens. (NASPHV 2006)

Culture positive for *M.tb* complex: Isolation and identification of *M. tuberculosis* complex organisms from any site using standard mycobacterial methods.

Culture positive (*M.tb* complex) elephant: An elephant from which a *M. tuberculosis* complex organism has been isolated from any body specimen. A culture positive elephant is considered positive until it has met the treatment requirements as outlined in the current Guidelines.

Dual Path Platform (DPP®) VetTB Assay: A new generation screening kit for the rapid detection of IgG antibodies to *M. tuberculosis* or *M. bovis* in elephant serum, plasma, or whole blood. The DPP® has shown 100% correlation with MAPIA™ (Greenwald et al. 2009).

ElephantTB STAT-PAK® Assay: A qualitative screening kit for the detection of antibodies to *M. tuberculosis* and *M. bovis* in elephant sera, plasma, or whole blood (Lyashchenko 2005, 2006, Greenwald 2009).

ELISA: Enzyme-linked immunosorbent assay; a test used to detect and measure either antigen or antibody.

Exposure: Risk of transfer of an infectious agent from a TB infected elephant(s) or contaminated environment through contact (direct, indirect) or airborne modes of transmission.

Fomite: An inanimate object or material on which disease-producing agents may be conveyed.

Gamma-interferon test: A whole blood *in vitro* assay that can be used as an ancillary diagnostic test for TB (not currently available for use in elephants).

Genotyping assay: A technique for the identification and analysis of polymorphism in certain types of repeat units in DNA. Restriction fragment length polymorphism (RFLP) and variable number tandem repeat (VNTR) are examples of genotyping techniques.

Herd: A group or groups of elephants, maintained on common ground. Alternatively, two or more groups of animals under common ownership or supervision that are geographically separated, but that may have an interchange or movement of animals or personnel without regard to health status.

Incidence: The rate at which a certain event occurs, for example, the number of new cases of a specific disease occurring during a certain period.

Index animal: The animal in which a disease is first diagnosed.

Infected elephant: an elephant from which *Mycobacterium tuberculosis* complex has been identified through culture, PCR or other molecular techniques or that is reactive on the ElephantTB STAT-PAK® Assay and the MAPIA™.

Intradermal tuberculin test (skin test): The injection of purified protein derivative (PPD) tuberculin into the skin for the purpose of detecting exposure to tuberculosis. In cattle, the test site is either the caudal fold (CFT) or cervical region (e.g. comparative cervical test, CCT) and the test is read by observation and palpation at 72 hours (plus or minus 6 hours) following injection. In humans, the test site is the forearm and the test is read at 48-72 hours. The intradermal tuberculin test is not a reliable test in elephants (Mikota 2001, Lewerin 2005).

Licensed veterinarian: a person who has graduated from an accredited school of veterinary medicine and who has a valid license to practice veterinary medicine in the U.S.

MultiAntigen Print ImmunoAssay (MAPIA™): A confirmatory test to the ElephantTB STAT-PAK® Assay for detection of antibodies to *M. tuberculosis* and *M. bovis* in elephant sera or plasma (Lyashchenko 2000, 2006, Greenwald 2009).

Mycobacteria other than TB (MOTT): See non-tuberculous mycobacteria.

Mycobacteriosis: A disease caused by non-tuberculous mycobacteria (NTM).

***Mycobacterium*:** A genus in the family Mycobacteriaceae.

Mycobacterium avium (M. avium): A non–tuberculous mycobacteria that is the primary causative agent of tuberculosis in birds. *M. avium* may be isolated from non-clinically affected elephants and is usually considered an environmental contaminant.

***Mycobacterium bovis (M. bovis)*:** The primary causative agent of tuberculosis in cattle, bison, and cervids; may also affect a variety of mammals including pigs, humans, primates, and non-domestic ungulates.

***Mycobacterium tuberculosis (M.tb*):** The primary causative agent of tuberculosis in humans; may also affect a variety of animals, including primates, pigs, cattle, dogs, parrots, elephants, and rhinos.

***Mycobacterium tuberculosis* complex (*M.tb* complex):** A group of mycobacteria which includes *M. tuberculosis, M. bovis*, *M. africanum*, *M. microti, M. canetti, M. caprae, and M. pinnipedii*. A vaccine strain derived from *M. bovis* (*M. bovis BCG*) is sometimes listed as a separate member of this complex.

Mycobacterium Tuberculosis Direct Test (MTD): A nucleic acid amplification test used in the diagnosis of TB. The MTD utilizes a technique that replicates RNA from bacteria of the *M. tuberculosis* complex.

No isolation: Absence of growth of *M. tb* complex organisms from trunk wash, feces, tissue or other samples using standard mycobacterial culture methods. Failure to isolate organisms may be due to the following reasons:
1. The animal is not infected
2. The animal was not shedding at the time of sample collection
3. Sampling error (culture overgrowth by contaminating organisms, inadequate sample, or laboratory error)
4. Improperly handled or shipped sample

Non-reactive: Absence of response; in the context of serological testing for TB in elephants, a non-reactive result indicates that an antigen-antibody reaction has not occurred in the presence of an appropriate positive control response.

Non-tuberculous mycobacteria (NTM): Mycobacteria that generally do not cause the formation of granulomas. Most NTM are saprophytes found in soil or water. They are typically non-pathogenic but may occasionally cause disease in humans and animals, including elephants. Also referred to as “atypical” mycobacteria or “Mycobacteria Other Than TB” (MOTT).

Nucleic acid amplification test: A technique that amplifies entities such as DNA or RNA.

PCR (polymerase-chain reaction): A nucleic acid amplification technique in which specific sequences of nucleic acid (DNA or RNA) are replicated, allowing for detection of target sequences.

Premises: A parcel of land containing elephants, administered by a person, government entity (city, county, state, region) or organization (zoological society, corporation).

Prevalence: The total number of cases of a specific disease in a given population at a given time.

Rapid Test: see ElephantTB STAT-PAK® Assay

Reactive: Presence of response; in the context of serological testing for TB in elephants, a reactive result indicates that an antigen-antibody reaction has occurred.

Report date: The date the laboratory reports the results.

Spoligotyping: A genotyping assay

Variable number tandem repeat (VNTR): A genotyping assay

Submission date: The date the sample is received at the laboratory.

Test date: The date the sample is collected.

Tested elephant: An elephant that has been tested for tuberculosis according to the protocol established in these guidelines.

Triple sample method: A method of culture collection whereby 3 samples are obtained on separate days.

Trunk wash: A procedure used in elephants to obtain a sputum sample using one of the approved methods outlined in Section 4 – Culture Collection Procedure.

Sensitivity: A measure of the ability of a test to identify infected animals. Sensitivity is the frequency of a positive or abnormal test result (e.g. a test that is outside of the reference interval) when a disease is present (i.e. the percentage of true positive results). Sensitivity = [TP ÷ (TP + FN)] X 100 where TP = true positive; FN = false-negative).

Specificity: A measure of the ability of a test to identify non-infected animals. Specificity is the frequency of a negative or "normal" test result when a disease is absent (i.e. the percentage of true-negative (TN) test results. Specificity = [TN ÷ (TN + FP)] X 100.

Untested elephant: An elephant is considered "untested" if it has not had three trunk washes obtained by the method outlined in this protocol within a 12 month period or if fewer than three valid culture results are obtained or if it has not been tested with the ElephantTB STAT-PAK® Assay performed by a USDA veterinarian trained and certified to perform the test.

3. ANNUAL TESTING

To adequately address the concerns of TB in the general elephant population, all captive elephants must be tested annually by culture and with the ElephantTB STAT-PAK® Assay (a blood test)**.** Samples for cultures and blood must be collected by or under the supervision of a licensed veterinarian according to current USDA requirements. Blood collection for the Guideline-required ElephantTB STAT-PAK® Assay must be witnessed by a federal or state veterinarian and performed as licensed by the USDA Center for Veterinary Biologics. See further information below under ElephantTB STAT-PAK® Assay. It is required that elephants with a reactive ElephantTB STAT-PAK® Assay result be tested using the confirmatory MultiAntigen Print ImmunoAssay (MAPIA™). See item 5 below**.**

Elephants should be tested within ± 30 days of the established annual test date. **Blood for ElephantTB STAT-PAK® Assay and culture should be collected within a 2 week period. All elephants must be tested every calendar year. Note that the date the sample is collected is the "test date," the date the sample is received at the laboratory is the "submission date," and the date the laboratory reports the results is the "report date."**

Record keeping of TB testing and treatment by the attending veterinarian is of upmost importance. It is recommended that attending veterinarians maintain open communication with the United States Department of Agriculture (USDA) and State Veterinarian, particularly concerning elephants under treatment for TB or in cases of exposure to TB positive elephants. It is recommended that at least a 1 ml aliquot of sera collected at the time of TB testing be sent to the elephant serum bank (See appendix 8).

4. CULTURE COLLECTION PROCEDURE (also see Appendix 3)

Samples for culture must be collected by or under the supervision of a licensed veterinarian using the "triple sample method." This method consists of obtaining three samples from the trunk on separate days. If possible, collect samples within a seven-day period. Do not pool samples. Samples should be taken after water has been withheld for at least two hours to reduce sample dilution and contamination. Light exercise prior to collection may facilitate obtaining secretions from lower in the respiratory tract, which is desirable. Of the following methods, the trunk wash with bag seems to provide the most effective way to collect samples at this time. **Samples collected by swab are not acceptable.** As there is a risk of human exposure to sputum produced during this procedure, personal protective measures are recommended for personnel during sample collection. These should include gloves and HEPA-filter masks certified by the National Institute for Occupational Safety and Health (NIOSH) to protect against TB (see Employee Health and Safety).

A. Trunk wash with bag (or other suitable container) - Using a catheter tip syringe, instill 60 ml sterile saline into the trunk. Raise the trunk as high as possible to distribute the fluid deeper into the trunk. Lower the trunk and place a clean, one-gallon plastic bag over the end of the trunk and hold in place until the elephant exhales into the bag. Transfer at least 20 ml of the sample to a sterile leak proof, screw-top container. Sterile 50-ml conical screw-top plastic centrifuge tubes are preferred and are available free of charge from the National Veterinary Services Laboratories (NVSL) – call 515-337-7388.

B. Trunk wash - Using a 14 French feeding tube, introduce 60 ml of sterile saline into the trunk then aspirate. Transfer at least 20 ml of the sample into sterile leak proof, screw-top container. Methods A and C are preferable to this method.

C. Forcible exhalation – Mucous collected without instilling saline into the trunk is acceptable if elephants are trained to forcibly exhale into a clean plastic collection bag and the volume collected is at least 20 ml. This may allow sampling of secretions from other areas of the respiratory tract and may be a preferable sample. Transfer the sample into sterile, leak proof, plastic screw-top container.

Storage

Do not expose samples to sunlight or heat. Consult receiving laboratory to determine whether samples should be refrigerated or frozen prior to shipment. For those laboratories that recommend freezing (i.e. NVSL) freeze samples as soon as possible after collection and keep frozen until shipment. Freeze at -20ºC (conventional freezer). As standard frost-free freezers undergo cyclic freeze-thaws to limit frost, freezers that do not have this feature are preferred. Freezing at -80ºC (ultra-low temperature freezer) is also acceptable. **Frozen samples must be shipped within 2 weeks of sample collection to the testing lab.**

Packaging and Shipping

All three refrigerated or frozen samples may be submitted together. **Label containers with the animal ID and date of collection** and put the same information on the submission form. Place screw-top containers in double zip-lock bags. **Do not send samples in glass containers or**

packaged only in plastic bags. Sterile 50-ml conical plastic centrifuge tubes with lids sealed with parafilm or electrical tape are preferred.

Place samples on ice packs or dry ice and ship overnight via Federal Express, Airborne, or other overnight carrier. **Do not ship by U.S. mail** as samples may be irradiated which will render them unacceptable. Packaging and shipping should be in accordance with the International Civil Aviation Organization Technical Instructions for the Safe Transport of Dangerous Goods by Air 2009-2010 (http://www.icao.int/icaonet/dcs/9284.html). Also helpful is the 2007 WHO document "Guidance on Regulations for the Transport of Infectious Substances (http://www.who.int/csr/resources/publications/biosafety/WHO_CDS_EPR_2007_2cc.pdf)

Packaging and shipping samples and cultures should be in accordance with Department of Transportation regulations – 49 CFR Parts 171, 172, 173 and 175- Hazardous Materials: Infectious Substances; Harmonization with the United Nations Recommendations; Final Rule, published June 2, 2006 in the Federal Register.
http://isu1.indstate.edu/terc/infectiousmaterial/pdf/Hazardous%20Materials_Infectious%20substances%20harmonization%20with%20the%20UN%20final%20rule.pdf
http://ecfr.gpoaccess.gov/cgi/t/text/text-idx?c=ecfr&rgn=div5&view=text&node=49:2.1.1.3.8&idno=49

Send samples to NVSL or other laboratory facility offering comparable procedures for identification of mycobacteria species. When submitting samples to NVSL, use VS Form 10-4, Specimen Submission Form. This form is available online in Word or pdf format: http://www.aphis.usda.gov/animal_health/lab_info_services/forms_publications.shtml.

Request mycobacterial culture with species differentiation.
Positive cultures from laboratories that do not have the capability to differentiate *M. tuberculosis* complex organisms must be forwarded to NVSL or other qualified laboratories for speciation. Culture of mycobacteria requires a minimum of eight weeks. **Laboratory reports that do not provide a definitive result due to contamination/overgrowth or other causes are considered invalid.** Additional samples should be collected and resubmitted to replace those reported as contaminated.

Note: Other mycobacteria species such as *M. avium*, *M. kansasii*, *M. elephantis,* and *M. fortuitum* have been isolated from elephants. At this time, there is no substantive evidence that these organisms are pathogenic for elephants. However, *Mycobacterium szulgai*, an unusual non-tuberculous mycobacterium, has been associated with pathology in elephants (Lacasse 2007).

5. ELEPHANTTB STAT-PAK® ASSAY SAMPLE COLLECTION PROCEDURE

Blood collection for the Guideline-required ElephantTB STAT-PAK® Assay must be witnessed by a federal or state veterinarian and performed as licensed. It is advisable to also bank a serum sample. Blood from elephants with reactive ElephantTB STAT-PAK® Assay results must be submitted for MAPIA™ /DPP® testing to:

Chembio Diagnostic Systems, Inc.
3661 Horseblock Road
Medford, NY 11763
Tel: 631-924-1135
Fax: 631-924-6033
Email: customerservice@chembio.com
Contact Chembio for shipping instructions.

The USDA veterinarian is responsible for shipping the sample but the owner must pay for shipping and must contact Chembio to arrange payment for the MAPIA™ or DPP® test.

6. ANCILLARY SCREENING / DIAGNOSTIC TESTS

A number of other ante mortem tests have been under investigation to diagnose TB in elephants. Following is a summary of those tests and current recommendations for their use.

Intradermal Tuberculin Test

A correlation between the intradermal tuberculin test (skin test) and culture results has not been established (Mikota 2001, Lewerin 2005). Therefore, intradermal tuberculin testing cannot be deemed reliable for screening or diagnosis and is not recommended.

Enzyme Linked Immunosorbent Assay (ELISA)

A multiple antigen ELISA was developed at the Animal Population Health Institute at Colorado State University (Larsen 2000). This test was used for detecting the presence of elephant serum antibodies to mycobacteria and investigations showed high sensitivity and specificity for detecting infected elephants and monitoring elephants over time. However, ELISA testing is not currently available.

Acid Fast Smears

Acid fast stains of trunk wash smears or other tissue are not reliable indicators of tuberculosis when used as a sole diagnostic test.

7. TB MANAGEMENT GROUPS (1-4)

All elephants will fall into one of four management groups (1-4) based on test results or will be untested (group 5). A culture positive elephant is defined as an elephant from which *Mycobacterium tuberculosis* or *Mycobacterium bovis* has been isolated from any body site or specimen. A culture positive elephant is considered positive until it has met the treatment requirements as outlined for Group 4. Exposure history has been incorporated into the Guidelines as ongoing data collection has indicated that it is an important risk factor. Flow charts are included in Appendix 9 to illustrate the management groups.

GROUP 1: Culture negative; ElephantTB STAT-PAK® non-reactive; no exposure to culture positive elephant in past 12 months.
Monitor annually by culture (triple sample method) and ElephantTB STAT-PAK® (single serum sample collected concurrently).

- No treatment or travel restrictions.
- No elephant should move into a facility where there is an untested elephant.
- If an elephant has had exposure to other untested elephants in the previous 3 months, then a STAT-PAK® test should be repeated in 3 months time to confirm. If the ElephantTB STAT-PAK® remains non-reactive, the elephant continues in Group 1.

GROUP 2: Culture negative; ElephantTB STAT-PAK® non-reactive; exposure to culture positive animal within the last 12 months.
Monitor by culture (triple sample method) and ElephantTB STAT-PAK® every 3 months for one year post-exposure, then every 6 months for 2 years, then annually thereafter if all cultures remain negative and ElephantTB STAT-PAK® remains non-reactive.

- No travel or public contact until 2 additional non-reactive ElephantTB STAT-PAK® tests are performed at 3 and 6 months post-exposure (6 month restriction).
    - If non-reactive at 6 months, travel/public contact restrictions removed as long as additional testing can be performed as outlined above.
- If the results during any of the follow-up testing change, the individual elephant will change group. No elephant should move into a facility where there is an untested elephant.

Note: The exact time to sero-conversion is unknown.

GROUP 3: Culture negative; ElephantTB STAT-PAK® reactive
It is required that blood from elephants with reactive ElephantTB STAT-PAK® results be submitted for MAPIA™ / DPP® testing (see item 5 above). Based on MAPIA™ /DPP® results and exposure history, the elephant will fall into one of the following subgroups:

A. Culture negative; STAT-PAK® reactive, MAPIA™/DPP® non-reactive, no known exposure
Monitor by culture (triple sample method) every 3 months for the first year after becoming ElephantTB STAT-PAK® reactive, then every 6 months for the next 2 years. Repeat MAPIA™ / DPP® every 6 months for the first year if elephant

remains STAT-PAK® reactive. If all cultures and MAPIA™/DPP® remain negative/non-reactive during this period, annual testing may resume.

- No treatment or travel restrictions.
- If the culture becomes positive or MAPIA™/DPP® becomes reactive during any of the follow-up testing the individual elephant will change category.
- No elephant should move into a facility where there is an untested elephant.

B. Culture negative; STAT-PAK® reactive, MAPIA™ /DPP® non-reactive, known exposure to TB culture positive elephant (no time limit on exposure history)

Monitor by culture (triple sample method) every 3 months for one year post-exposure, then every 6 months for two years then annually thereafter if all cultures remain negative. Repeat MAPIA™/DPP® every 6 months for the first 3 years if elephant remains STAT-PAK® reactive. If all cultures and MAPIA™/DPP® remain negative/non-reactive during this period, annual testing may resume after 3 years.

- No travel or public contact for first year; if results are unchanged at the first year, restrictions are removed.
- If the culture or MAPIA™/DPP® results change during any of the follow-up testing and become positive, the individual elephant will change group.
- Culture positive elephants that have completed a course of anti-tuberculosis therapy may remain ElephantTB STAT-PAK® reactive and fall into this group. If appropriate treatment has been documented and approved by USDA, these animals will not have travel/public contact restrictions unless there is a change to positive culture and/or reactive MAPIA™/DPP® results during follow-up testing.

C. Culture negative; STAT-PAK® reactive, MAPIA™/DPP® reactive, no known exposure

Monitor by culture (triple sample method) every 3 months for one year, then every 6 months for life. Repeat MAPIA™/DPP® every 3 months for the first year, then every 6 months for an additional 2 years if elephant remains STAT-PAK® reactive. If all cultures remain negative after 3 years annual serological testing may resume as described in these guidelines.

- No travel or public contact until the first year of testing has been completed.
- Treatment should be considered. If serological conversions are demonstrated to be recent (within the past 12 months then prophylactic treatment can be used. If serological conversions are longer standing or unknown, then full treatment may be advisable. Individual cases should be evaluated in conjunction with USDA. If treatment is performed, the elephant may be able to travel and have public contact after 6 months of successful documented USDA approved treatment.
- If the culture or MAPIA™/DPP® results change during any of the follow-up testing the individual elephant will change group.

Note: The STAT-PAK® and MAPIA™ /DPP® tests have been shown to be early indicators of TB infection. Retrospective studies have shown elephants may be serologically reactive months to years in advance of detection by culture (Greenwald 2009).

D. Culture negative; STAT-PAK® reactive, MAPIA™/ DPP® reactive, known

exposure to TB culture positive elephant (no time limit on exposure history)
Monitor by culture (triple sample method) every 3 months for one year post-exposure, then every 6 months for life. Repeat MAPIA™/DPP® every 3 months for the first year, then every 6 months for an additional 2 years if elephant remains STAT-PAK® reactive. If all cultures remain negative after 3 years, annual serological testing may resume as described in these Guidelines.

- No travel or public contact until the first year of testing has been completed.
- Treatment should be considered. If serological conversions are demonstrated to be recent (within the past 12 months) then prophylactic treatment can be used. If serological conversions are longer standing or unknown then full treatment may be advisable. Individual cases should be evaluated in conjunction with USDA. If treatment is performed, the elephant may be able to travel and have public contact after 6 months of successful documented USDA approved treatment.
- If the culture or MAPIA™/DPP® results change during any of the follow-up testing the individual elephant will change group.
- Culture positive elephants that have completed a course of anti-tuberculosis therapy may remain ElephantTB STAT-PAK® reactive and fall into this category. If appropriate treatment has been documented and approved by USDA, these animals will not have travel/public contact restrictions unless there is a change in their results during follow-up testing. It has been shown that the MAPIA™/DPP® will decline and may indicate a response to treatment so on-going annual monitoring with MAPIA™/DPP® is required for life as changes in MAPIA™ may detect relapse.

Considerations for ElephantTB STAT-PAK® reactive elephants.

Elephants may develop antibodies to mycobacterial antigens months to years prior to detection by culture, however, the time intervals between exposure, seroconversion, and shedding are not precisely known. Numerous variables such as age, genetics, immune status, nutritional condition, other concurrent health problems, and other factors influence the development of disease in an individual animal following exposure to a pathogenic agent. Results of MAPIA™/DPP® testing are useful in helping determine potential risk categories as defined above and determine which animals require more frequent surveillance or should undergo prophylactic treatment (Greenwald 2009).

There may be a possible association with chronic inflammatory conditions, such as arthritis, in elephants that are ElephantTB STAT-PAK® reactive, but non-reactive on MAPIA™/DPP® and with no known TB exposure based on a small number of cases. Review history for possible exposure to a culture positive animal or previous treatment for TB since this may also affect results. Nonetheless, it is important to monitor these elephants for possible development of infection and disease. Retrospective analyses of banked serum samples are strongly encouraged to provide a more complete serological history.

Elephants that are culture negative, ElephantTB STAT-PAK® reactive and MAPIA™/DPP® reactive are at increased risk of either latent or active TB. Factors to consider in the decision to administer treatment vs. increased monitoring include exposure history, age, whether the elephant travels, potential exposure of personnel or public, side effects of treatment, concurrent health problems, etc. Increased monitoring and travel/public contact restrictions is required based

on risk. If culture results during any of the follow-up testing become positive, the individual elephant will move to Category 4.

Consideration should be given to minimizing or eliminating contact with the public that would result in exposure by contact or aerosol transmission and to providing personal protective equipment such as a NIOSH certified N95 respirator /N95 face mask for staff when working in close proximity to elephants that are under enhanced surveillance. Employees must be respirator fit tested before they use the N95 respirator.

Based on a history of exposure to a culture positive animal, or other considerations, the attending veterinarian may elect to administer prophylactic or full treatment after consultation with USDA.

Effective prophylactic therapy is defined as the administration of a specific number of doses of two anti-TB drugs within a specified time. It must be demonstrated that adequate anti-TB drug levels are achieved in the blood of the elephant under treatment. Acceptable anti-tuberculosis drugs include isoniazid (INH), rifampin (RIF), pyrazinamide (PZA), ethambutol (ETH), or a fluoroquinolone such as levofloxacin, moxofloxacin, ciprofloxacin, or enrofloxacin. Isoniazid is recommended as one of the two drugs if a known exposure case isolate is INH sensitive. PZA should not be given if *M. bovis* infection is suspected since this organism is inherently resistant to PZA.

Prophylactic therapy is for 9 months can be administered using either of the following schedules:

Prophylactic Treatment Schedule 1 (preferred):
Administer two anti-TB drugs daily for 9 months (270 total doses). The first 60 doses should be administered within a period of 90 days (i.e. no more than 30 days of "refused medication" should occur. <u>It must be documented that the elephant received 270 total doses at a dosage level sufficient to achieve adequate drug serum levels.</u>

Prophylactic Treatment Schedule 2:
Administer the two anti-TB drugs daily for two months (as above, the first 60 doses should be administered within a period of 90 days). Adequate levels of both drugs must be demonstrated in two serum samples collected approximately two weeks apart. <u>Serum samples should be collected as soon as the elephant is accepting medication reliably. If acceptable levels (see below) are not achieved, the dosage should be adjusted and serum levels tested again (two samples collected approximately two weeks apart). It must be documented that the elephant received the first 60 doses at a dosage level sufficient to achieve adequate drug serum levels.</u> Once this has been demonstrated, administer the two drugs every other day but at twice the previous dosage level for an additional 9 months (105 total doses of every other day dosing plus the initial 60 doses for a total of 165 doses). It is not necessary to repeat serum drug levels when changing to the every other day schedule.

Note: Pyridoxine 50 mg is administered to humans receiving INH for treatment of active or latent tuberculosis to prevent the development of peripheral neuropathy. Although this side effect has not been reported in elephants, it may be possible. At the discretion of the attending

veterinarian, Vitamin B6 (pyridoxine) can be given prophylactically at a dose of 0.8-1 mg/kg daily.

Concomitant use of INH, rifampin, and PZA with other hepatotoxic drugs should be done with caution.

Refer to TB Drugs section for starting dosages, routes of administration, side effects, blood levels, and other information.

Monitoring of Prophylactically Treated Elephants
During the 9 months of treatment, elephants should be closely observed for changes in appetite, behavior, and any other signs that may be attributable to adverse drug effects. Monthly blood tests (CBC and serum chemistry profile) are recommended to monitor general health and possible drug effects on the liver. Liver tests (AST, ALT, LDH, bile acids, and bilirubin) should be included in the serum chemistry panel. Isoniazid may cause hepatitis and anemia. In addition, leukopenia has occurred in at least one elephant apparently due to INH toxicity).

GROUP 4: *M. tuberculosis* complex positive culture
Animals that have had *Mycobacterium tuberculosis* complex isolated from any sample (sputum, stool, tissue, etc.) are considered culture positive for TB. A culture positive elephant is defined as an elephant from which *Mycobacterium tuberculosis* complex organism has been isolated from any body site or specimen.

The ElephantTB STAT-PAK® and MAPIA™/DPP® tests must be performed on blood from culture positive elephants. Serum for MAPIA™/DPP® testing must be submitted regardless of ElephantTB STAT-PAK® results.

Positive cultures must be submitted to NVSL for genotyping.

A culture positive elephant is considered positive until it has met the treatment requirements as outlined below. These elephants must be separated from the public for the duration of the treatment period. Separation from previously non-exposed elephants is also recommended until treatment is completed. Precautions to safeguard personnel health and safety should be instituted immediately (see Employee Safety and Health section). Elephants with cultures that yield non-tuberculous strains of mycobacteria are not considered infected and are not a risk to other animals or humans. Options for Category 4 elephants include:

Options:
A. Treatment: This is the preferred option for culture positive elephants whenever possible.
1. If the organism was isolated at a laboratory other than NVSL and they do not perform mycobacterial species differentiation and DNA fingerprinting, the owner must request that the laboratory submit the isolate to NVSL or other qualified laboratory for mycobacterial species differentiation and DNA fingerprinting.

2. Antimicrobial sensitivity testing should be performed on all positive isolates. Sensitivities should be requested for the following drugs: isoniazid, rifampin, pyrazinamide, ethambutol,

ciprofloxacin (or other fluoroquinolone), and amikacin. (Antimicrobial susceptibility testing for *M. tuberculosis* complex organisms is now available at NVSL).

3. Perform ElephantTB STAT-PAK® and MAPIA™ every 3 months during treatment then every 6 months for 2 years then according to the schedule in the group that the elephant falls into post-treatment. Serological monitoring of treated elephants with MAPIA™ has shown changes that may indicate successful treatment or recrudescence of infection (Lyashchenko 2006).

4. Beginning with the onset of treatment, cultures should be collected by the triple sample method every 2 months for the first 6 months of treatment, then every 6 months for the remainder of the elephant's life. This intensive screening by culture ensures adequate therapy during the treatment period and after treatment has ended to ensure that the animal does not revert to a positive culture, which would again pose a risk to animals or humans.

5. Pending antimicrobial susceptibility results, initiate empiric therapy with 3 or 4 of the following drugs: isoniazid, rifampin, pyrazinamide, and ethambutol or a fluoroquinolone (moxifloxacin is preferred). Following the human model, initiating empiric treatment with four drugs is considered "ideal." However, the difficulties associated with training an elephant to accept medications are acknowledged. After determining sensitivities, continue treatment using one of the following schedules:

Schedule 1 (preferred): Administer 3 drugs to which the isolates are susceptible daily for 2 months. The first 60 doses should be administered within a period of 90 days (i.e. no more than 30 days of "refused medication" should occur). Adequate blood levels of all 3 drugs must be demonstrated in two samples collected approximately two weeks apart. Serum samples should be collected as soon as the elephant is accepting medication reliably. If acceptable levels (see below) are not achieved, the dosage should be adjusted and serum levels tested again (two samples collected approximately two weeks apart). It must be demonstrated that the elephant received the first 60 doses at a dosage level sufficient to achieve adequate drug serum levels. Treatment is then continued daily for an additional 10 months with 2 drugs to which the isolate is susceptible for a total number of doses (with two drugs) of 300. As above, the inclusion of INH is recommended. The total number of doses for the entire treatment is 360. The entire treatment should be completed within 15 months (this allows for "refused medicine" days and periods of interruption that may be needed if side effects are noted).

Schedule 2: Administer 3 drugs to which the isolate is susceptible for 2 months. The first 60 doses should be administered within a period of 90 days (i.e. no more than 30 days of "refused medication" should occur). Adequate levels of all drugs must be demonstrated in two samples collected approximately 2 weeks apart. Serum samples should be collected as soon as the elephant is accepting medication reliably. If acceptable levels (see below) are not achieved, the dosage should be adjusted and serum levels tested again (two samples collected approximately two weeks apart). It must be demonstrated that the elephant received the first 60 doses at a dosage level sufficient to achieve adequate drug serum levels. Continue treatment with two drugs at twice the dosage used in the initial period every other day for 10 months (150 doses). It is not necessary to repeat serum drug levels. The total number of doses is 210. The entire treatment should be completed within 15 months (this allows for "refused medicine" days and

periods of interruption that may be needed if side effects are noted). Animals that have not completed treatment are considered as non-treated.

Note: Peripheral neuropathy can sometimes occur in humans receiving INH. Although this side effect has not been reported in elephants, it may be possible. At the discretion of the attending veterinarian, Vitamin B6 (pyridoxine) can be given prophylactically at a dose of 1 mg/kg daily.

Travel: Elephants in Group 4 should not travel or have public contact (direct or indirect) until treatment is completed according to the guidelines.

Additional Monitoring of Treated Elephants
Elephants should be closely observed for changes in appetite, behavior, and any other signs that may be attributable to adverse drug effects. Monthly blood tests (CBC and serum chemistry profile) are recommended to monitor general health and possible drug effects on the liver. Liver tests (AST, ALT, LDH, bile acids, and bilirubin) should be included in the serum chemistry panel. Isoniazid may cause liver damage and anemia. In addition, leukopenia has occurred in at least one elephant apparently due to INH toxicity).

B. Quarantine without treatment: This option may be considered especially for animals that are already housed alone and not considered a good candidate for treatment (ex. bull elephant). Additional precautions must be taken for human safety (such as the use of N-95 masks, gloves, etc). Quarantined elephants should be kept out of range from non-infected animals and should be monitored for signs of TB disease.

- No travel is permitted.
- No public contact that would result in exposure by contact or aerosol transmission is permitted.
- No exposure to other elephants is permitted.
- Additional testing (trunk wash culture, ElephantTB STAT-PAK®/MAPIA™/DPP®), ancillary tests and nucleic acid amplification are recommended for data collection.

C. Euthanasia: This option may be considered for those animals that are showing clinical signs considered to be poor candidates for treatment, or for other factors based on the clinician's discretion. A thorough postmortem examination must be performed (see section 11).

Group 5: Untested If an elephant cannot complete procedures as outlined for official annual testing, it should not be permitted to have public contact that would result in exposure by contact or aerosol transmission, or contact with other tested elephants (or their enclosures or equipment). Untested elephants should not be moved from their home facilities. A tested elephant should not move into a facility housing an untested elephant unless it can be demonstrated that there will be no direct contact with the untested elephant or with its enclosure or equipment. If a tested elephant(s) is in contact or housed with an untested elephant, the tested elephant cannot travel nor have public contact until the untested elephant is tested unless approved by USDA.

8. PRINCIPLES OF ANTI-TUBERCULOSIS THERAPY

The American Thoracic Society has published guidelines for the treatment of tuberculosis in humans (see references). In brief, it is necessary to treat active TB with multiple drugs to prevent the emergence of resistant strains of bacteria. For individuals exposed to TB (positive skin test), but no signs of active disease (negative chest radiograph, negative sputum cultures), treatment is typically with a single drug (INH).

The guidelines for the treatment of TB in elephants are based on the assumption that animals with known active disease are treated similarly to humans. However, for elephants, the treatment period has been extended. For a category 3 elephant with negative cultures and presumed exposure based on positive serologic response, i.e., positive ElephantTB STAT-PAK® (and MAPIA™), treatment is a "modified" regime – with two drugs for 9 months. Skin testing is not reliable in elephants. Acid-fast smears are not reliable on elephant trunk washes.

For humans, treatment of primary tuberculosis is to empirically administer 4 first line drugs while waiting for antimicrobial sensitivity testing. This assures that initial treatment includes at least 2 drugs to which the organism is susceptible. And, the additional number of antibiotics results in more rapid clearance of bacteria from the sputum thereby decreasing the public health risk.

Once susceptibility tests are received, and the sputum has reverted to being smear negative, the number of drugs is decreased to two first line drugs for the remainder of treatment. When the index case is known, and the index isolate is known to be susceptible to all anti-mycobacterial drugs, then initial treatment may be limited to three drugs. However, in the vast majority of cases the index case is not known with certainty and four drugs are given. Moreover, in regions or situations when the frequency of resistance exceeds 10%, empiric initial therapy for humans consists of five drugs.

The length of therapy for humans is currently 6 months for active tuberculosis. This includes the initial period of 3-5 drugs as above and 2-drugs for the remainder of treatment. For individuals with resistance to a single antibiotic, treatment is extended to 12 months with 2 drugs to which the organism is susceptible. For individuals infected with multi-drug resistant tuberculosis (MDR-TB), treatment is for at least 12 months with 2-4 drugs based on the susceptibility pattern (lower numbers of agents are employed if the isolate is susceptible to INH or rifampin). Because the long term outcome and efficacy of treatment for TB of non-human species is currently unknown, treatment of elephants is structured for a 12-month course.

9. ANTI-TUBERCULOSIS DRUGS

Antituberculous agents are divided into first and second line agents. First line agents include isoniazid, rifampin, pyrazinamide, ethambutol, and streptomycin. These are agents with the greatest activity and the best side effect profiles. Second line agents include those with less activity and/or greater side effects. Second line agents include capreomycin, ethionamide, cycloserine, and thiacetazone. The fluoroquinolones (FQ; moxifloxacin, ciprofloxacin, levofloxacin, and enrofloxacin) while not considered as 1st line agents have significant bactericidal activity against *M. tuberculosis*. Moreover, published studies report the equivalency of FQ substitution for ethambutol in the treatment of TB in humans and studies are underway to investigate FQ use for the treatment of latent TB infection. Linezolid, a drug active against Gram positive bacteria such as *Staphylococcus aureus*, MRSA, enterococcus, and VRE has also been shown to have significant activity against M. tuberculosis and has been used successfully in salvage regimens. Amikacin, an aminoglycoside (as is streptomycin), is a mainstay in the treatment of non-tuberculous mycobacterial infection and has been used in salvage regimens against MDR-TB. Pharmacokinetic studies of INH, RIF, EMB, and PZA in elephants have been published (Maslow et al. 2005a, Maslow et al. 2005 b, Zhu et al. 2005, and Peloquin et al. 2006).

FIRST LINE AGENTS

Isonicotinic acid hydrazide (Isoniazid, INH)
Mechanism of action: INH acts to inhibit cell wall synthesis through blockage in the mycolic acid pathway. The specific target enzymes are unknown; however, evidence supports a role for the catalase enzyme, *katG*, as modifying INH to an active form. Postulated targets of the activated form of INH include ketoacyl synthetase and inhA.

Metabolism and excretion: INH is acetylated in the liver through the action of *N*-acetyl-transferase. The acetylated product is then excreted in the urine. Some ethnic groups (Native Americans, Eskimos, and Orientals) as well as others carry a recessive allele encoding for rapid acetylation of INH those results in more rapid clearance and lower bioavailability. It is not known whether elephants are polymorphic in this enzyme and differ in the speed of acetylation.

Toxicity: The major adverse effects documented in humans are hepatitis (principally hepatocellular inflammation with a transaminitis) and peripheral neuropathy. Uncommon adverse reactions include headaches, optic neuritis, seizures, psychosis, encephalopathy, twitching, rashes, and gastrointestinal upset. A histamine like reaction can be observed when products with tyramine (red wine, cheese) are ingested. Risk factors for hepatic toxicity in humans include age greater than 35 yr, concomitant viral hepatitis (Hepatitis B or C), and other hepatic toxins (drugs, alcohol). Vitamin B6 (pyridoxine) is given at a dose of 50 mg daily (~1 mg/kg) to prevent the development of peripheral neuropathy.

Toxicity in elephants: Observed toxicities of INH have included inanition, transaminitis, and anemia. Fermented products (mash or other feeds) should likely be avoided to minimize potential histamine reactions. Liver values (SGOT, SGPT, and bilirubin) should be monitored monthly for 2 months and then bimonthly if no liver toxicity is observed. INH has caused

irreversible leukopenia in camels; reversible leukopenia has been observed in one elephant that was considered as possibly / probably related to INH.

Route of administration: In humans INH is administered orally. In elephants, INH is preferentially administered as an oral bolus. However, rectal absorption is efficient, yielding levels similar to oral bolus dosing. In bongo antelope, INH has also been successfully administered via intramuscular injection.

Rifampin (RIF)
Mechanism of action: Rifampin is a semi synthetic derivative of rifamycin, an antibiotic derived from the fungus *Streptomyces mediterranei*. Rifampin acts to inhibit the DNA-dependent, RNA-polymerase thus blocking formation of messenger RNA (the first step in protein synthesis).

Metabolism and excretion: Rifampin is acetylated in the liver. Both the unaltered and acetylated drug is excreted into the bile. Rifampin is then reabsorbed whereas the acetylated form is not.

Toxicity: The major toxicity of rifampin is hepatitis. Other side effects include gastrointestinal upset, renal failure, hemolysis, acute renal failure, and thrombocytopenia. It is avoided in pregnancy during the first trimester because of possible teratogenicity.

Rifampin is also a strong inducer of the cytochrome P450 hepatic enzymes that may increase the metabolism of concurrently administered drugs. A prime example is exogenously administered steroids used for in vitro fertilization. For animals being treated for other conditions, potential drug-drug interactions should be ruled out.

Toxicity in elephants: The toxicity in elephants is unknown. Similar adverse reactions to humans should be expected. Therefore it is recommended that in addition to liver tests, serum creatinine, electrolytes and CBC be monitored per the schedule listed for INH.

Route of administration: Rifampin is administered to humans orally although intravenous administration is used in patients unable to tolerate oral dosing. In elephants rifampin appears to be absorbed well as an oral bolus although acceptance is low because of the drug's bitterness. Rifampin is not absorbed rectally; there is no known experience with parenteral administration in elephants or other animals. Urine and feces may become orange colored while on this drug.

Pyrazinamide (PZA)
Mechanism of action: Pyrazinamide is a synthetic antibiotic derived from nicotinic acid. Its mechanism of action is unknown; however the presence of an intact pyrazinamidase is required. Since *Mycobacterium bovis* lacks this enzyme, it is resistant to PZA.

Toxicity: Toxicities observed in humans include arthralgias and arthritis, hyperuricemia, hepatitis, gastrointestinal upset, and photosensitivity (skin rashes).

Toxicity in elephants: The toxicity for elephants is unknown, however hepatitis may have been observed. Similar adverse effects as documented for humans should be expected.

Route of administration: In humans, pyrazinamide is administered orally. In elephants both oral and rectal dosing have yielded acceptable blood levels. Pyrazinamide has been successfully administered to bongo antelope via subcutaneous injection.

PZA is should not be given if M bovis infection is suspected since this organism is inherently resistant to PZA."

Ethambutol (EMB)
Mechanism of action: Ethambutol is a specific inhibitor of the arabinosyl transferase thereby inhibiting formation of arabinogalactose and lipoarabinomannan, which are the dominant lipids in the *M. tuberculosis* cell wall.

Toxicity: The major toxicity of ethambutol is optic neuritis, which may result in decreased visual acuity, a central scotoma, and loss of red-green discrimination. Ethambutol may also cause peripheral neuropathy, headache, rashes, arthralgias, hyperuricemia, and rarely anaphylaxis.

Toxicity in elephants: The toxicity for elephants is currently unknown.

Route of administration: Ethambutol is administered orally to humans and elephants. Rectal administration is irritating and poorly tolerated resulting in expulsion of the drug. Subcutaneous administration has been given successfully to bongo antelope.

Streptomycin
Mechanism of action: Streptomycin is an aminoglycoside antibiotic derived from the fungus *Streptomyces griseus* that acts on the 30S ribosome to inhibit protein synthesis.

Toxicity: Similar to other aminoglycosides, streptomycin administration may result in auditory-vestibular and renal toxicity. Specific symptoms include ataxia, vertigo, nerve deafness, and renal failure. Most symptoms are reversible if the drug is discontinued immediately after their occurrence.

Toxicity in elephants: The toxicity for elephants is currently unknown but is likely the same as for humans.

Route of administration: Streptomycin is administered via intramuscular injection to humans. There is no experience in administering streptomycin to elephants.

SECOND LINE AGENTS

Fluoroquinolones: Moxifloxacin, Ciprofloxacin, Levofloxacin, Enrofloxacin

Mechanism of action: Fluoroquinolone antibiotics act to inhibit the topoisomerases DNA gyrase and topoisomerase IV. Both of these enzymes are needed during DNA replication to first

unwind supercoiled DNA and then to again achieve a supercoiled structure of DNA. Of the commercially available fluoroquinolones, moxifloxacin has the greatest in vitro activity and in vivo activity in a mouse model of infection followed by ciprofloxacin and levofloxacin (Neurmberger EL et al, Moxifloxain-containing regimens of reduced duration produce a stable cure in murine tuberculosis, Am J Respir Crit Care Med 2004, 170: 1131-4). The anti-tuberculous activity of enrofloxacin, a derivative of ciprofloxacin is unknown. Gatifloxacin also has excellent in vitro activity against strains of TB, although the drug was recently withdrawn due to reports of antibiotic associated diarrhea and QT-prolongation. Studies are underway examining the role of Moxifloxacin in standard treatment and prophylaxis regimens (Burman et al. Moxifloxacin versus ethambutol in the first 2 months of treatment for pulmonary tuberculosis. Am J Respir Crit Care Med 2006, 174: 331-8; Pletz MW et al. Early bactericidal activity of moxifloxacin in treatment of pulmonary tuberculosis: a prospective, randomized study, Antimicrob Agents Chemother 2004, 48: 780-2).

Toxicity: The quinolone antibiotics may result in arthropathy, cartilage defects in adolescent animals, photosensitivity, antibiotic related diarrhea, and electrocardiographic prolongation of the QT interval.

Toxicity in elephants: The toxicity for elephants is unknown.

Route of administration: These agents are administered either orally or intravenously (levofloxacin only). Oral levofloxacin has been administered to bongo antelope, although poor serum levels were observed. Oral levofloxacin has been used to successfully treat a *Klebsiella spp*. infection of the hock in a horse. (J Maslow, personal communication). Enrofloxacin has been used to treat one elephant with disseminated multi-drug resistant TB as part of a multi-drug regimen. The animal developed photo-induced blepharitis, although this adverse effect had been episodic during infection and was initially detected prior to the institution of enrofloxacin. Thus, the causal association to enrofloxacin is unknown.

Amikacin

Mechanism of action: Amikacin is an aminoglycoside antibiotic that acts on the 30S ribosome to inhibit protein synthesis. Isolates that are resistant to streptomycin may be susceptible to amikacin.

Toxicity: Similar to other aminoglycosides amikacin administration may result in auditory-vestibular and renal toxicity. Specific symptoms include ataxia, vertigo, nerve deafness, and renal failure. Most symptoms are reversible if the drug is discontinued immediately after their occurrence.

Toxicity in elephants: The toxicity for elephants is currently unknown but is likely the same as for humans.

Route of administration: Amikacin is administered via intravenous injection to humans. Amikacin has been administered via intramuscular injection to bongo antelope yielding acceptable serum levels (unpublished). A pharmacokinetic study of amikacin in African elephants has been conducted (Lodwick, L.J., Dubach, J.M. and Phillips, L.G., 1994.

Pharmacokinetics of amikacin in African elephants. J Zoo Anim. Med 25: 367-375). There is no published information regarding amikacin in Asian elephants. Amikacin in one Asian elephant given IM 3 times a week at 14 mg/kg yielded good blood levels (acceptable levels in elephants unknown) and was eliminated almost completely from serum within 72 hours. However, significant toxicity occurred with prolonged use of this drug at this dose (personal communication, Dr. G Dumonceaux).

Other second line agents have not been used for mycobacterial infections in elephants. Clinicians contemplating the use of agents other than those listed should consult with the USDA on an individual basis.

The four first-line drugs used to treat tuberculosis in humans are isoniazid (INH), rifampin (RIF), pyrazinamide (PZA) and ethambutol (ETH). Second-line drugs used in cases of drug intolerance or multi-drug resistant organisms include amikacin and a fluoroquinolone. Both fluoroquinolones and linezolid have been used in cases of multidrug resistance in humans (Veziris, N. et al. Fluoroquinolone-containing third-line regimen against Mycobacterium tuberculosis in vivo. Antimicrob Agents Chemother 2003, 47: 3117-22).

10. DOSAGES AND ROUTES OF ADMINISTRATION

Anti TB drugs must be directly administered. Placing drugs over food does not produce reliable blood levels and this is not an acceptable method of treatment. Drugs vary in palatability and acceptance so some experimentation may be required to determine a workable regimen for each individual elephant.

Isoniazid and PZA can be given either orally or rectally. Rifampin and ethambutol should only be administered orally (effective blood levels of rifampin cannot be achieved with rectal administration and ethambutol is quickly expelled when given rectally). Below are suggested starting doses, but actual doses may need to be adjusted in order to achieve adequate blood levels and / or reduce effects of toxicity.

Drug	Dosage (mg/kg)	Route	Formulation	Target conc (µg/ml)	Cmax (hr)
Isoniazid	5	Oral	premixed suspension	3-5	1-2
	4	Oral	Powder	3-5	0.5-1
	4	Rectal	premixed suspension	3-5	0.25-0.5
Rifampin	10	Oral only	Powder	8-24	2-4
Pyrazinamide	30	Oral or rectal	Powder	20-60	1-2
Ethambutol	30	Oral only	Powder	2-5	1-2

The dosages quoted above are based primarily on the pharmacokinetic studies of drug administration to the first herds of treated elephants as reported (Maslow et al 2005a, Maslow et al 2005b, Zhu et al 2005, Peloquin et al 2006). Recent studies have demonstrated that INH achieves Cmax much more quickly than previously thought when administered rectally Dosages are considered as estimates with the goal of achieving target serum concentrations as listed in #10 below without causing significant side effects that interrupt treatment. Serum drug levels or drug side effects may dictate that dosages be adjusted up or down accordingly. Sequential MAPIA™ tests may also be used to monitor response to treatment (Lyashchenko 2006). Second line agents should only be considered and administered following consultation with the facility USDA inspector.

11. BLOOD LEVELS

Target blood levels for elephants treated with each of the anti-tuberculosis drugs are based on the experience in humans. Target serum concentrations are listed in the table above. Blood levels approximating those found in humans have been reported for elephants with each of the four 1st line agents INH, RIF, PZA, and EMB (Maslow et al 2005a, Maslow et al 2005b, Zhu et al 2005, Peloquin et al 2006).

Blood levels should be determined to measure the maximal concentration of drug (Cmax). While INH, PZA, and EMB are rapidly absorbed with a Cmax occurring between 1-2 hrs, drug absorption may vary between elephants and may also vary drug to drug. Recent studies have demonstrated that INH achieves Cmax much more quickly than previously thought when administered rectally. Importantly, the time to Cmax (Tmax) may vary over the course of treatment due to multiple factors such as food intake, drug acceptance, etc. Thus, at the start of treatment and periodically through the course of therapy it is important to measure drug levels at multiple time points until Cmax for each drug and animal is determined.

For INH, PZA, and EMB it is recommended that drug levels be determined at 1hr, 1.5hr, and 2 hr and for RIF at 2hr, 3hr, and 4hr except if INH is administered rectally and then 15 min and 30 min blood levels are recommended to accurately measure the Cmax. If the first measured time point represents the greatest level for any drug, then Tmax may have already passed and earlier time points should be assessed. Conversely, if the last measured time point represents the greatest concentration for any drug, then Tmax may occur later than the range chosen and later time points should be assessed. During the initial phase of treatment, time ranges should always be assessed to determine the true Tmax.

NOTE: Target blood levels for anti-TB drugs in elephants have not been rigorously established. Until further studies can be conducted, target blood levels of anti-TB drugs for elephants must necessarily be based on human data. Although achieving blood levels comparable to humans is the ideal goal, the attending veterinarian should be aware that there is unpublished evidence that some elephants cannot tolerate anti-TB drugs at the doses required to achieve the above levels. Isoniazid, in particular, has caused side effects. It may be necessary to reduce the dose of an anti-TB drug to eliminate side effects, which may result in lower blood levels. The attending veterinarian should carefully document observed side effects, dosage changes and associated anti-TB drug levels in these cases. Variations to these Guidelines require consultation with the facility USDA inspector.

12. POSTMORTEM EXAMINATION

It is essential that a post-mortem examination be performed on all elephants that die. The examination must include a thorough search for lesions of tuberculosis regardless of exposure status. A comprehensive elephant necropsy protocol has been prepared by the Elephant SSP and is available at these websites:

www.elephanttag.org
www.elephantcare.org

Prior to any planned euthanasia of an elephant, trunk washes, blood for serology and any other ancillary tests should be performed regardless of whether or not TB is suspected. In this way, valuable data can be gathered to evaluate the efficacy of the current testing protocol. In the event of a sudden death, collect post-mortem blood and separate serum for other tests.

It is recommended that a trained veterinary pathologist direct the necropsy if possible. In the event of an elephant necropsy (elective or otherwise), contact Dr. Scott Terrell (Elephant SSP Pathology Advisor) for further instructions and possible participation:

Scott P. Terrell, DVM, Diplomate ACVP, SSP Pathology Advisor, Disney's Animal Kingdom, 1200 N Savannah Circle, Bay Lake, FL 32830, W (407) 938-2746; H (407) 251-0545; Cell (321)229-9363; email Scott.P.Terrell@disney.com

The following information is excerpted from the SSP Elephant Necropsy Protocol:

Protective equipment for tuberculosis cases - Mandatory
Respiratory protective equipment should be available during any elephant necropsy procedure regardless of the historical TB testing status of the animal. In animals with an unknown, suspect, or positive TB test history, respiratory protection should be considered **mandatory**. OSHA standards (29CFR1910.134) require that "workers present during the performance of high hazard procedures on individuals (humans) with suspicious or confirmed TB" be given access to protective respirators (at least N-95 level masks).

Similar precautions should be taken during an elephant necropsy. According to the draft CDC guidelines for the prevention of transmission of tuberculosis in health care settings, respiratory protective devices used for protection against *M. tuberculosis* should meet the following criteria:

1. Particulate filter respirators approved include (N-, R-, or P-95, 99, or 100) disposable respirators or positive air pressure respirators (PAPRs) with high efficiency filters)
2. Ability to adequately fit wearers who are included in a formal respiratory protection program with well-fitting respirators such as those with a fit factor of greater than or equal to 100 for disposable or other half-mask respirators
3. Ability to fit the different face sizes and characteristics of wearers. This can usually be met by supplying respirators in at least 3 sizes. PAPRs may work better than half-masks for those persons with facial hair.

Consult these websites for OSHA and CDC guidelines:

1. OSHA TB standards and rules: http://www.osha.gov/SLTC/tuberculosis/standards.html

2. Guidelines for Preventing the Transmission of *Mycobacterium tuberculosis* in Health-Care Settings, 2005: http://www.cdc.gov/nchstp/tb/Federal_Register/New_Guidelines/TBICGuidelines.pdf

Necropsy procedures

All elephants undergoing necropsies should have a careful examination of the tonsillar regions and submandibular lymph nodes for tuberculous appearing lesions. These lymph nodes may be more easily visualized following removal of the tongue and laryngeal structures during the dissection. All lymph nodes should be carefully evaluated for lesions since other sites may also be infected (ex. reproductive or gastrointestinal tract). Collect any nodes that appear caseous or granulomatous for mycobacterial and standard bacterial culture (freeze or ultrafreeze), and fixation (in buffered 10% formalin). In addition, search thoracic organs carefully for early stages of TB as follows: after removal of the lungs and trachea, locate the bronchial nodes at the junction of the bronchi from the trachea. Use clean or sterile instruments to section the nodes. Freeze half of the lymph node and submit for TB culture to NVSL or a laboratory experienced in mycobacterial culture and identification (**even if no lesions are evident**). Submit sections in formalin for histopathology. Carefully palpate the lobes of both lungs from the apices to the caudal borders to detect any firm B-B shot to nodular size lesions. Take **NUMEROUS (5 or more)** sections of any suspicious lesions. Open the trachea and look for nodules or plaques and process as above. Regional thoracic and tracheal lymph nodes should also be examined and processed accordingly. Split the trunk from the tip to its insertion and take samples of any plaques, nodules or suspicious areas for TB diagnosis as above. Look for and collect possible extra-thoracic TB lesions, particularly if there is evidence of advanced pulmonary TB.

13. EMPLOYEE HEALTH AND SAFETY

All employees that are in direct contact with elephants should be tested for TB annually following established human testing guidelines. New employees should be tested prior to contact with elephants.

Any employee with a positive intradermal test (i.e. a positive intradermal reaction to purified protein derivative (PPD) of *M. tuberculosis*) should be evaluated for the possibility of active TB. It is recommended that health care providers who manifest a positive PPD receive INH prophylaxis unless there is a contraindication to treatment. Conversely, those declining treatment are followed yearly with a chest radiograph and clinical evaluation to determine whether they have developed active disease.

A positive skin test may result from either exposure to *M. tuberculosis*, *M. bovis*, BCG injection, or exposure to non-tuberculous strains of mycobacteria. The American Thoracic Society has published guidelines for the interpretation of intradermal testing. If inoculation with BCG occurred more than 10 years ago, a positive PPD test should not be considered a reaction due to BCG, but should instead be considered as positive for exposure to TB.

Employees with acid-fast positive sputum smears should be removed from animal contact until it is determined whether this represents infection with an organism of the *M. tuberculosis* complex (*M. tuberculosis* or *M. bovis*). Treatment guidelines and recommendations for contact with animals and humans are available through state public health departments. At the present time there is no known transfer of non-tuberculous strains of mycobacteria between humans and animals (or human to human) via aerosolization or any other route and thus, there are no restrictions placed on animals or humans known to be colonized or infected such organisms.

Any facility housing a known culture-positive (*M. tuberculosis* complex*)* animal should develop a program to protect employees from TB exposure, to include the use of appropriate face masks (N95 HEPA filtered masks, certified by the National Institute for Occupational Safety and Health to protect against TB), disinfection procedures, and the use of separate implements for infected animals. The local public health department should be contacted for further guidelines.

Measures to protect staff from infected animals should include the use of respiratory (N95) HEPA filtered masks during all direct or indirect contact with infected animals, such as cage cleaning, medication administration, feeding, watering, etc. The facility should contact local health agencies and should provide additional other protective gear such as gowns, gloves, etc.

No specific precautions are necessary for animals that are culture positive for mycobacteria other than *M. tuberculosis* and *M. bovis*.

Best practices for the safe conduct of work in biomedical and clinical laboratories and animal facilities in regards to *Mycobacterium tuberculosis* are listed in the 5th Edition of Biosafety in Microbiological and Biomedical Laboratories published by the U.S. Department of Health and Human Services in 2007. http://www.cdc.gov/od/ohs/biosfty/bmbl5/BMBL_5th_Edition.pdf

14. REPORTING

Tuberculosis is a reportable disease. Positive culture results must be reported to the State Veterinarian and appropriate public health agencies.

15. APPENDICES

APPENDIX 1. REFERENCES CITED AND ADDITIONAL READING

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APPENDIX 2. ACKNOWLEDGMENTS

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APPENDIX 3. A TRUNK WASH TECHNIQUE FOR THE DIAGNOSIS OF TUBERCULOSIS IN ELEPHANTS

Ramiro Isaza, DVM, MS and Cornelia Ketz, DVM

Summary

A trunk wash is a practical method of collecting a sample from an elephant's distal respiratory tract for Mycobacterium culture and is the technique recommended in the "Guidelines for the Control of Tuberculosis in Elephants" by the National Tuberculosis Working Group for Zoo and Wildlife Species. The procedure, however, is potentially dangerous to the handlers and requires cooperation of the elephant. Because of the limitations of using culture results as a screening test, the trunk wash results should be interpreted with care. A positive culture result identifies an elephant that is shedding tuberculosis organisms whereas a negative result is non-diagnostic.

Introduction

Tuberculosis in Asian elephants (*Elephas maximus*) has been sporadically reported in the literature for many years (1, 2). The isolation of *Mycobacterium tuberculosis* from elephants in the United States has resulted in the development of the "Guidelines for the Control of Tuberculosis in Elephants" by the National Tuberculosis Working Group for Zoo and Wildlife Species (http://www.aphis.usda.gov/ac/ElephTBGuidelines2000.html). Compliance with this policy requires that all elephants have annual mycobacterial cultures. In these guidelines, the trunk wash is recommended as the most practical method of obtaining a culture sample from an elephant. This paper describes the trunk wash technique as the authors are currently using it.

Materials and methods

The trunk wash technique requires that the elephant allow the handlers to restrain and manipulate the tip of trunk. This is difficult in an untrained elephant in that most elephants resent this manipulation, and the trunk is many times stronger than the combined force of several handlers. It is therefore important that the animals be trained to present the trunk, allow gentle manual restraint, and manipulation of the trunk tip during the collection of the sample. The training period varies with the individual elephant, the prior behavioral conditioning of the animal, and the skill of the handlers. In our experience, most animals can be adequately trained for the procedure in 2-4 weeks.

The materials needed for a trunk wash include: Sterile 0.9% saline solution, sterile 60 ml syringe, 1 gallon plastic zip lock type bags (heavy duty), and sterile, 50 ml, screw top, plastic jar or centrifuge tube. As long as attention is given to collecting a clean sample from the distal nasal passages, the materials and techniques for the sample collection can be modified. For example, some clinicians prefer to use a 14-gauge red rubber tube feeding tube inserted into the trunk tip instead of simply flushing the sterile saline into the trunk tip. Another common variation is to use a sterile plastic container to catch the trunk wash fluid instead of a plastic bag.

Procedure

A routine screening of an elephant should consist of a series of three trunk wash samples collected on separate days within a one-week period. Trunk washings should be collected in the morning and prior to water being offered to the animal. These recommendations are made in an

attempt to obtain a representative sample of the nasal flora from the previous night, and to avoid the dilution effect caused by elephants drinking water with their trunks.

The elephant's trunk is manually restrained by the handlers so that the tip is held up. The 60 ml syringe filled with sterile saline is then inserted into one of the nostrils and the saline quickly flushed into the trunk. The handler then lifts the trunk tip as high as possible to help the fluid flow as far into the trunk as possible. The 1 gallon plastic bag is then slipped over the trunk tip and the tip of the trunk is lowered to allow the fluid to drain. If possible, the elephant is allowed to exhale into the bag during this collection phase of the procedure. A good sample should retrieve a significant portion of the saline that was placed into the trunk (about 40 ml). The sample should contain visible mucus from the inside of the trunk and often contains dirt and food particles that are normally found inside the trunk. The collection of moderate amounts of foreign material does not invalidate the sample. If, however, the collector feels the contamination is excessive, a second flush may be attempted.

Once the sample is collected in the plastic bag, it is carefully transferred into a labeled container. Ideally, the sample is refrigerated and sent directly to a laboratory for processing and mycobacterial culture. If the sample cannot be sent directly for culturing, it may be frozen in a regular freezer (-20 to -10 ^{O}C) until it can be sent to the laboratory. Often the recommended three daily cultures samples are collected and frozen until all samples are collected and the batch of samples can be sent to the laboratory together.

Discussion

Identification of a *M. tuberculosis* infected animal has significant management implications to both the animal and the collection. Management of the infected animal may require isolation of the exposed herd, potential removal of the animal from exhibit or shows, and if elected, treatment of the animals and exposed herd which can be very expensive. In the worst case, a positive diagnosis may lead to euthanasia of the infected animals. For these reasons, the screening test selected needs to be definitive and have as few false positives as possible. A positive culture of *M. tuberculosis* is, therefore, the only diagnostic test result used as a basis for making decisions in the guidelines.

The trunk wash as a method of collecting a culture sample from elephants was selected by the National Tuberculosis Working Group for Zoo and Wildlife Species because it is a practical method of obtaining a culture sample from a large proportion of the elephant population. The procedure requires no sedation or undue stress to the animal. Additionally, the procedure requires no specialized or expensive equipment.

An important consideration of this procedure is that it can potentially be very dangerous to the handlers. This is particularly true when attempted on an uncooperative elephant, because any attempts to manually restrain the trunk in an uncooperative elephant can lead to injury. The time spent training the elephant to accept this method will greatly increase the efficiency and safety of the procedure. In some cases, with potentially dangerous or unpredictable animals, an increased level of handler safety can be obtained by having the animal lie in sternal or lateral recumbency prior to sample collection. This technique does not guarantee safety or successful sample collection, as it still requires cooperation of the animal and does not replace adequate training. In

the case of elephants managed under protective contact, the animal's trunk can be handled though a set of bars. This method still requires that the animal is fully cooperative and, therefore, usually requires extensive training prior to the collection.

A second safety issue is the potential for zoonotic infection. Recently there has been documentation of a zoonotic transmission of tuberculosis between humans and elephants (3). During the collection of the trunk wash sample, there is exposure to aerosolized mucus from the elephant's respiratory tract. The authors, therefore, suggest that the collectors and handlers wear protective gear during the collection process. Minimal precautions would include a well fitted respirator or face mask capable of filtering 0.3 micron particles, disposable gloves, and working in a well-ventilated, sunlit, area.

Mycobacterial culture as the primary method of detecting infected animals has several limitations that are best illustrated by examination of the underlying biological assumptions. The first assumption is that most infected elephants have respiratory infections. Although the literature suggests that most infected elephants have respiratory infection, there have been no comprehensive necropsy studies to confirm these observations. The second assumption is that most infected animals shed mycobacterial organisms into the respiratory tract. There is little data that determines if and when an infected animal will begin shedding organisms. It is unknown what proportion of elephants can carry latent or "walled off" infections that would be missed with culturing techniques. A third assumption is that animals that are shedding will pass mycobacteria organisms at least once in the three-day testing period. Currently it is unknown if shedding animals pass organisms periodically or continuously. Finally, the samples collected from the distal trunk are often contaminated with normal bacterial flora and foreign material. It is assumed that these contaminants do not routinely overgrow or mask the growth of pathogenic mycobacteria, although no studies have tested this assumption. The interpretations of the culture results should, therefore, be limited. A positive culture is strong evidence that the animal is shedding mycobacteria and is infected; negative culture results provide little information as to whether the elephant is infected or not.

Culturing the distal trunks of all the animals in a population will only detect animals shedding tuberculosis through the trunk, and not detect all animals that are infected. However, with time and repeated cultures of all animals in the population, it may be possible to detect and treat most of the elephants shedding infectious organisms. If these animals are then treated properly and shedding of organisms stops, the spread of tuberculosis from elephant to elephant should decrease in the population.

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APPENDIX 4. TESTING LABORATORIES

CULTURES, ANTIMICROBIAL SENSITIVITY, GENOTYPING

USDA APHIS VS
National Veterinary Services Laboratories (NVSL)
1920 Dayton Avenue
Ames, IA 50010
Lab web site: http://www.aphis.usda.gov/animal_health/lab_info_services/diagnos_tests.shtml

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Send trunk washes to NVSL either frozen or on icepacks by overnight express (Federal Express handles diagnostic samples). Containers should be leak proof and double-bagged (50 ml conical screw-top centrifuge tubes are preferred) and are available free of charge from NVSL.

If lesions are submitted for culture, tissues should be frozen and sent on ice packs overnight. Lesioned tissues should be split and ½ should be sent to the histopathology lab so PCR can be run to see if the tissue is compatible for tuberculosis. There is no charge for histopathology on lesioned tissue.

Use the VS Form 10-4 for submission, not the VS 6-35 form found in the TB kit. If the formalized tissue is sent separately from the frozen tissue, please indicate on the submission forms that there are 2 separate packages coming from the same animal so that the reports can be combined and accession numbers coordinated when they reach NVSL. It is also helpful to call or email NVSL contacts when sending TB suspects to schedule testing and relay any relevant history of the case.

NVSL Trunk wash cost: $98 per sample for processing which includes a Gen Probe® DNA probe on any isolate. If the sample is positive for mycobacteria and speciation is requested, the charge is $122.00 per sample which includes biochemical analysis, 16s rDNA sequencing analysis, spoliotyping and VNTR genotyping. DNA fingerprinting of *M. tuberculosis* or *M. bovis* isolates is also available. Antimicrobial susceptibility testing is available for *M. tuberculosis*

complex organisms for $112.00 per isolate. Please contact NVSL at (515) 337-7388 for test schedule.

To establish an account at NVSL for billing, contact **Connie Osmundson** (515) 337-7571 or Email: Connie.J.Osmundson@aphis.usda.gov .

(User fees as of October 1, 2010). Call lab before shipping samples for current prices and schedule of testing or check prices at the NVSL web site:
http://www.aphis.usda.gov/animal_health/lab_info_services/diagnos_tests.shtml

Mycobacteriology Laboratory at National Jewish Medical and Research Center
National Jewish Medical and Research Center
Director: Leonid Heifets, M.D.
1400 Jackson St.
Denver, CO 80206
(303) 398-1384
E-mail: heifetsl@njc.org
For price list, shipping instructions, and requisition form:
http://www.nationaljewish.org/research/clinical-labs/about/learn/mycobac/index.aspx
Serum sample submission: it is important to protect the samples from light by wrapping the tubes in tinfoil and to separate the serum and freeze it without delay, transferring the serum to a tube or cryovial that is also wrapped in tin foil. Samples should be sent on dry ice as well.

HISTOPATHOLOGY

Scott P. Terrell, DVM, Diplomate ACVP
SSP Pathology Advisor
Disney's Animal Kingdom
1200 N Savannah Circle
Bay Lake, FL 32830
W (407) 938-2746;
H (407) 251-0545;
Cell (321) 229-9363;
Email Scott.P.Terrell@disney.com
Send sections in formalin of any gross lesion and complete set of tissues including lung, liver, spleen, mesenteric lymph nodes, bronchial lymph nodes and other major organs. Use leak proof container.

USDA APHIS NVSL Pathobiology Laboratory
1920 Dayton Avenue
Ames, IA 50010
(515) 337-7521
Fax (515) 337-7527
Lab web site: http://www.aphis.usda.gov/animal_health/lab_info_services/diagnos_tests.shtml

Dr. Art Davis
Director of Pathobiology Laboratory
(515) 337-7526
Email: Arthur.J.Davis@aphis.usda.gov

Dr. Mark Hall
Head Pathological Investigations
(515) 337-7927
Email: Mark.Hall@aphis.usda.gov

Send formalin sections of any gross lesion and target tissues (lung, liver, mesenteric and bronchial lymph nodes). Use leak proof container. **Please indicate on submission form if a sample was submitted for culture so that the testing can be coordinated and results combined on one form.**

ANTI TB DRUG LEVELS

Infectious Diseases Pharmacokinetics Laboratory (IDPL)
National Jewish Medical and Research Center
1400 Jackson St.
Denver, CO 80206
Web site: http://www.nationaljewish.org/research/clinical-labs/about/learn/infectious/idpl.aspx

Refer to the above website for specimen handling instructions and to download Requisition forms.

Infectious Disease Pharmacokinetics Lab, College of Pharmacy, and Emerging Pathogens Institute
Charles Peloquin, Pharm.D.
Professor and Director
University of Florida
1600 SW Archer Rd., Rm P4-33
PO Box 100486
Gainesville, FL 32610-0486
Tel: 352-273-6266
Fax: 352-273-6804
peloquin@cop.ufl.edu
Call or email for information on sample submission.

The National Veterinary Services Laboratories
USDA APHIS NVSL
1920 Dayton Avenue
Ames, IA 50010
Web site: http://www.aphis.usda.gov/animal_health/lab_info_services/diagnos_tests.shtml

Dr. David Kinker
Head, Serology Section
515-337-7950
Email: David.R.Kinker@aphis.usda.gov
Call before shipping samples for current prices.

Chembio Diagnostic Systems, Inc.
3661 Horseblock Road
Medford, NY 11763
Tel: 631-924-1135
Fax: 631-924-6033
Email: customerservice@chembio.com
Call Chembio before shipping samples for current prices on veterinary products such as ElephantTB STAT-PAK®, MAPIA™ or DPP®.

APPENDIX 5. USDA Standard Operating Procedure for Processing Elephant Trunk Washes for the Isolation of Mycobacteria

United States Department of Agriculture
National Veterinary Services Laboratories

Standard Operating Procedure
Processing Elephant Trunk Washes for the Isolation of Mycobacteria

Mention of trademark or proprietary product does not constitute a guarantee or warranty of the product by USDA and does not imply its approval to the exclusion of other products that may be suitable.

1. Purpose

The purpose of this Standard Operating Procedure (SOP) is to describe the procedure for processing elephant trunk washes for the isolation of *Mycobacterium tuberculosis* used in the Mycobacteria and Brucella (MB) section.

Warning: *Mycobacterium bovis, M. tuberculosis and M. avium* are pathogenic to humans and they are a Class III pathogen. All procedures must be performed in a Class II or Class III biological safety cabinet.

2. Materials

2.1 50 ml sterile conical centrifuge tubes
2.2 15 ml sterile conical centrifuge tubes
2.3 Sorvall RC 3BP refrigerated centrifuge
2.4 N-Acetyl-L cysteine (NALC); Sigma catalog number A-7250
2.5 NaOH-Sodium citrate solution; (NVSL #10687)
2.5.1.1 Dissolve 29 gm of sodium citrate dehydrate in 1000 ml of Super Q H_2O. Dissolve 40 gm of sodium hydroxide pellets in 1000 ml of Super Q H_2O. Combine the 2 solutions and dispense as requested. Autoclave for 15 minutes at 121 °C.
2.6 Sterile distilled water
2.7 Johne's antibiotic mixture (contains vancomycin, amphotercin B, and nalidixic acid); NVSL #20215
2.7.1 Brain Heart Infusion Broth (NVSL #10009) 18.5 g
2.7.1.1 Combine 37 gm of Difco BHI w/out dextrose (BBL # 250220) with 1000 ml Super Q H_2O. Bring to a rolling boil. Dispense as requested. Autoclave 20 min. at 121°C for flasks, 15 min. for tubes.
2.7.2 Super QH_2O 1000 ml
2.7.3 Nalidixic Acid (NVSL #40153) 10 ml
2.7.3.1 Mix 10 g of nalidixic acid with 500 ml of distilled H_2O. Add 10N NaOH drop by drop until solution clears and QS to 1000 ml. Filter

sterilize solution thru a 0.22µm filter into sterile jug with bell end. Dispense (wearing gloves), 20 ml into a 50 ml sterile conical tube. Caution – chemical is carcinogenic.

2.7.4 Vancomycin (NVSL #40151) 10 ml

2.7.4.1 Combine 9.346 gm with 1000 ml of distilled Super Q H_2O and mix well. Filter sterilize. Dispense into 50 ml sterile tubes in 20.5 ml amounts.

2.7.5 Amphotericin B (NVSL #40154) 5 ml

2.7.5.1 Add 10 ml warm sterile distilled H_2O to a 100 mg Amphotericin B (Fungizone). Shake gently until dissolved and dispense as requested.

2.7.6 Combine BHI broth and Super QH_2O. Autoclave for 20 min at 121°C. Cool to 50°C. Remove 25 ml of cooled broth and discard. Add Nalidixic Acid, Vancomycin, and Amphotericin B. Mix well. Dispense in tubes and cover with foil because the Amphotericin B is light sensitive. Store in -20°C freezer. Media is good for 3 months.

2.8 37ºC CO_2 incubator preferred

2.9 Media set-up (one tube of each per sample):

2.9.1 Middlebrook 7H10 w/glycerol; NVSL #10941 or BBL Middlebrook and Cohn 7H10 Agar tubes (BBL #220959).

2.9.2 Middlebrook 7H11 w/glycerol; NVSL #10942 or BBL Seven H11 Agar tubes (BBL #221392) or BBL Selective Seven H11 Agar tube (BBL #297639).

2.9.3 Stonebrinks; NVSL #10451

2.9.4 Mycobactosel L-J medium (BBL #221414)

2.9.5 Bactec 12B medium vial with Panta and Erythromycin (32 µg/ml)

2.10 1 ml tuberculin syringes

2.11 5 ml syringes

2.12 Slant trays, media tube baskets

2.13 Vortex

2.14 Sterile swabs

3. Procedures

3.1 Carefully pour 10 – 12 ml of the trunk wash into a 50 ml conical centrifuge tube. If there is less than 10 ml, use the entire sample.

3.1.1 At this time, also pour 10 - 12 ml of sterile distilled water into a 50 ml conical centrifuge tube; this sample will be labeled “negative control” and will be processed the same as the rest of the samples.

3.2 Pour 10 to 12 ml (or whatever is left if < 10 ml) of the remaining trunk wash into a 15 ml conical centrifuge tube for storage.

3.2.1 These 15 ml centrifuge tubes are stored at -20 ± 2º C for a minimum of 8 weeks or until the bacterial isolation procedure is completed.

3.2.2 Samples from cases in which no isolation was made are retained in a -20º $\pm$ 2 ° C freezer for at least 6 months.

3.2.2.1 Samples that have no isolation and are older than 6 months can be discarded by the procedure in the current version of MBSOP0008.

3.2.3 Samples from cases in which mycobacteria have been isolated will be retained for one year and stored in a -20º C freezer.

3.3 Allow the 10 - 12 ml of the trunk wash in the 50 ml conical tube to stand undisturbed for 15 – 20 minutes to allow sediment to settle to the bottom. Or Alternately:
Pulse spin the 10 - 12 ml of trunk wash in the 50 ml centrifuge to spin down excess sediment. This can be accomplished by centrifuging for 1 minute and 40 seconds at 3000RCF, 10ºC, using the Sorvall RC 3BP centrifuge.

3.4 Slowly pour the supernate, trying not to disturb the sediment, into a sterile 50 ml conical centrifuge tube.

3.5 Prepare the N-Acetyl-L-cysteine (NALC)/NaOH- sodium citrate solution according to the following proportions:

Volume (ml)	NaOH- sodium citrate [a] (ml)	NALC (g)
50	50	0.25
100	100	0.50
150	150	0.75
200	200	1.00
300	300	1.50

a. 1:1 mixture of 4% NaOH to 2.9% sodium citrate
b. Allow NALC to dissolve in solution before use
c. Discard this solution after 24 hours

3.6 Add an equal amount of the NALC solution to the trunk wash supernate up to a maximum of 10 ml using a sterile pipette.

3.6.1 Be careful to avoid cross contamination of samples when adding the NALC solution.

3.7 Vortex or vigorously hand shake for 20 $\pm$ 5 seconds

3.8 Routinely allow the trunk wash to remain in contact with the NALC for 15 $\pm$ 5 minutes.

3.8.1 If the sample is extremely cloudy or appears to be contaminated, the NALC solution may need to remain in contact with the sample for up to 20 $\pm$ 5 minutes.

3.8.2 NALC is a mucolytic agent and this procedure reduces or eliminates contaminating bacteria while releasing Mycobacteria which may be trapped in mucin and cells, allowing them to grow. Observe the tube for clearing before proceeding to the next step.

3.9 Add enough sterile distilled water to the NALC/wash solution to fill the centrifuge tube

3.10 Centrifuge the water/NALC/wash solution for 20 minutes at 6000g and 10ºC.

3.11 Carefully pour off and discard the supernatant.

3.12 Re-suspend the sediment with 2 mls of sterile distilled water and 1 ml of the Johne's antibiotic mixture.

3.12.1 Vortex the re-suspended mixture for 20 ± 5 seconds.

3.13 Incubate overnight at 37 ± 2ºC.

3.14 Vortex or vigorously hand shake the incubated sample for 20 ± 5 seconds.

3.15 Using a sterile swab per specimen, swab the sample over the entire surface of each of the solid media tubes listed in section 2 of this SOP.

3.15.1 The four solid media tubes are Middlebrooks 7H10 w/glycerol, Middlebrooks 7H11 w/ glycerol, Mycobactosel LJ, and Stonebrinks.

3.15.2 If one of these media is not available, contact the section head or their designate for which media to be substituted.

3.16 Prepare the BACTEC 12B bottles.

3.16.1 A 5 ml syringe is used to add 2 ml of Erythromycin to the reconstituting fluid supplied for the PANTA solution for a final concentration of 32 µg/ml.

3.16.2 Using a new 5 ml syringe, add 5 ml of the reconstituting fluid to the vial of PANTA.

3.16.3 Using a 1 ml tuberculin syringe, each vial of the BACTEC media is inoculated with 0.2ml of the reconstituted supplement.

3.17 Inoculate the BACTEC media with your sample.

3.17.1 Using a 1 ml tuberculin syringe, inoculate the BACTEC media with ≤ 0.5 ml of the sample

3.18 Place the inoculated solid media on a slant rack and incubate overnight at 37 ± 2ºC in 10% CO_2, if available.

3.18.1 Incubation in an atmosphere of CO_2 will encourage earlier growth.

3.18.2 After being incubated overnight on a slant rack, the media tubes can be stored in the 37ºC incubator in an upright position.

3.19 Place the inoculated BACTEC bottles in a locked 37ºC incubator.

3.20 Read the media tubes as outlined below and record the results on the appropriate worksheets:

3.20.1 Solid Media

- 3.20.1.1 Read tubes weekly for weeks 1-7 after inoculation.
- 3.20.1.2 For tubes in week 8, read and record the results and discard the tubes if there is no suspicious growth for Mycobacteria noted in the tubes.
- 3.20.1.3 For tubes that contain contamination, discard the media tubes that are overgrown. If the entire set of media is overgrown, the case may need to be retested.

3.20.2 BACTEC Bottles

- 3.20.2.1 BACTEC bottles are read twice weekly for the first 3 weeks and then once a week until week 6.
- 3.20.2.2 Results of the growth indicator values are recorded on the BACTEC worksheet assigned to the case on the bottle each time the BACTEC bottle is read by the BACTEC 460 machine.

3.20.2.3 BACTEC bottles that have a growth indicator value of 300 or higher are examined by acid fast staining. See the current version of MBSOP2210 for these procedures. BACTEC bottles that are to be discarded are stored in a locked cabinet in the biological safety level 3 laboratories until they are removed by the personnel from Environmental Health and Safety. See the current version of MBSOP2007 and MBSOP2008 for these procedures.

4. References

4.1 Della-Latta, P. and I. Weitzman. 1998. Mycobacteriology. p. 169-204 In H.D. Isenberg (ed.), *Essential Procedures for Clinical Microbiology.* American Society for Microbiology, Washington, D.C.

4.2 Zimbro, M.J. and D.A. Power. 2003. Difco & BBL Manual – Manual of Microbiological Culture Media. Becton, Dickinson and Company, Sparks, MD.

APPENDIX 6. CONTACTS FOR QUESTIONS

DIAGNOSIS AND TREATMENT

Michele A. Miller, DVM, MS, PhD
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Cell: 931-628-5962
Email: smikota@elephantcare.org
Website: www.elephantcare.org

ELEPHANT TB STAT–PAK® ASSAY AND MAPIA™

Konstantin Lyashchenko, Ph.D.
Research Director, Mycobacterial Immunology
Chembio Diagnostic Systems, Inc.
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Tel: 631-924-1135, ext.111
Fax: 631-924-6033
Email: klyashchenko@chembio.com

REGULATORY

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Field Specialist for Elephants
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HUMAN HEALTH, ELEPHANT THERAPY AND TREATMENT

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NECROPSY, PATHOLOGY

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Home: 407-251-0545
Cell: 321-229-9363
Email: Scott.P.Terrell@disney.com

INTERNET

These guidelines are available on the Internet at the following sites:

1. http://www.aphis.usda.gov/animal_welfare/index.shtml (available to the public)
2. www.aazv.org (available to AAZV members by password)
3. www.elephantcare.org (available to the public)
4. www.elephanttag.org (available to the public)

APPENDIX 7. SOURCES FOR ANTI-TUBERCULOSIS DRUGS

There are various veterinary compounding pharmacies that have experience with formulations for elephants. Please contact one of the consultants in Appendix 6 for information. Select veterinary compounding pharmacies are also listed on www.elephantcare.org.

APPENDIX 8. Elephant Serum Bank Submission Form

Institution/owner: _____

Submitter: _____
Address: _____
_____
Tel: _____ Fax: _____ Email: _____

Animal Information
Asian [ ] African [ ] ISIS# _____ Studbook # _____
Name _____ Age: _____ [ ] actual [ ] estimate
Sex: [ ] male [ ] female

SAMPLE COLLECTION INFORMATION
Date of sample collection: _____ Time of collection: _____
Site of sample collection: [ ] ear vein [ ] leg vein [ ] other: _____
Health status of animal: [ ] normal [ ] abnormal
Fasted: [ ] no [ ] yes – how long _____
Weight _____ [ ] actual [ ] estimated
Type of restraint: [ ] manual [ ] anesthetized/sedated [ ] behavioral control
Temperament of animal: [ ] calm [ ] active [ ] excited
Type of blood collection tube:
[ ] no anticoagulant (red-top)
[ ] EDTA (purple)
[ ] heparin (green)
[ ] other: _____
Sample handling: [ ] separation of plasma/serum by centrifugation
(Check all that apply) [ ] stored as whole blood
[ ] frozen plasma
[ ] other – describe _____

TB EXPOSURE STATUS
[ ] Known infected animal
[ ] Known exposure to culture positive source within the past 12 months
[ ] Known exposure to a culture positive source within the past 1-5 years
[ ] No know exposure to a culture positive source in the last 5 years

TREATMENT INFORMATION
Is elephant currently receiving any medication or under treatment? [ ] yes [ ] no
If yes, please list drugs and doses: _____
_____
_____
_____
Time between blood collection and last treatment: _____

Ship samples overnight frozen with shipping box marked “PLACE IN FREEZER UPON ARRIVAL”

Send completed form with samples to:
Michele A. Miller, DVM, MS, PhD
Chief Veterinary Officer and Director of Conservation Medicine
Palm Beach Zoo, 1301 Summit Blvd., West Palm Beach, FL 33405
Phone: 561-833-7130 ext 224; Cell: 561-727-9630;
Email: mmiller@palmbeachzoo.org

Consent Form for Use of Serum by Elephant SSP

I give consent for the serum submitted to the Elephant Species Survival Plan (SSP) serum bank to be used for research on any elephant related issues based on recommendations by the veterinary advisor and/or steering committee.

The results could be reviewed and used by the SSP veterinary advisor in providing health-related recommendations and publications.

I understand that all results and recommendations regarding the individual elephant will be kept confidential.

_____ Yes, I agree to allow the SSP to use our sample for designated research and testing results.

_____ No, I do not consent to the use of our sample and test results unless specified.

_____ _____
Signature, title Date

_____ _____
Printed name Phone number

_____ _____
Institution Email address

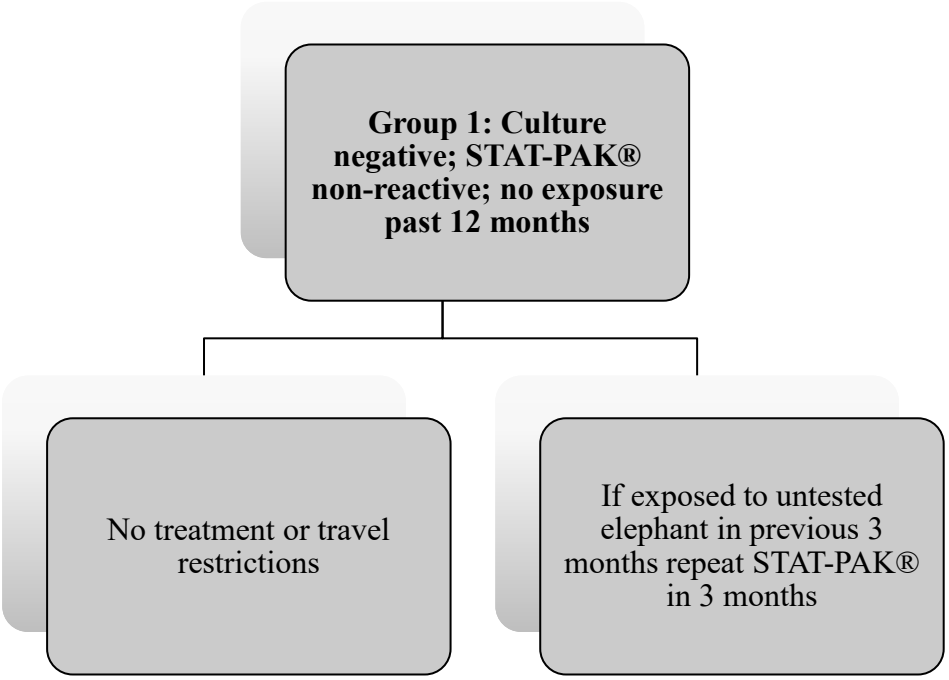
_____
Address
_____

_____

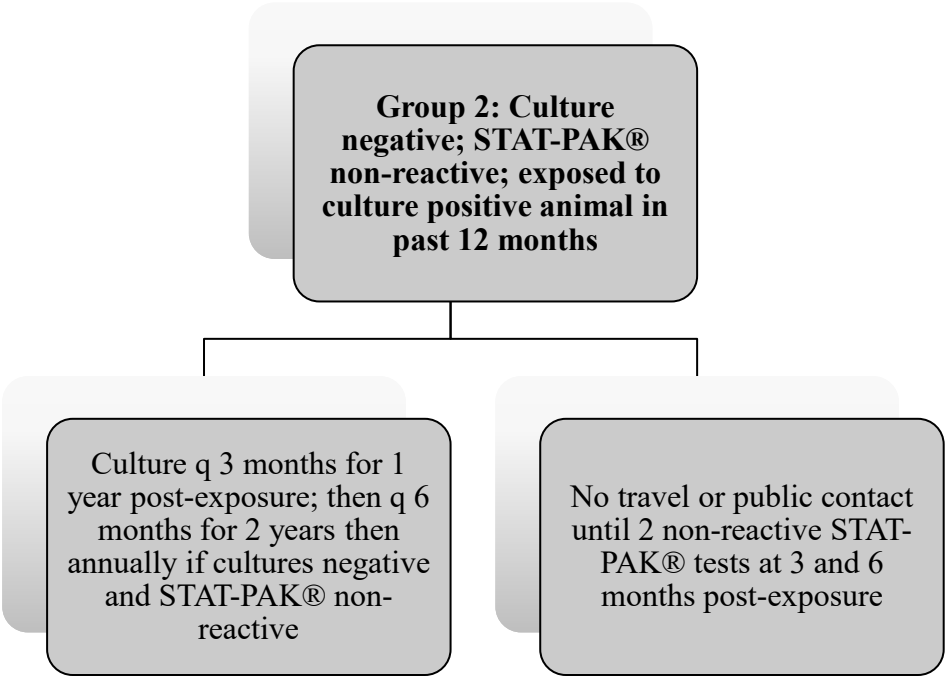
Comments: _____

Appendix 9. TB Management Groups

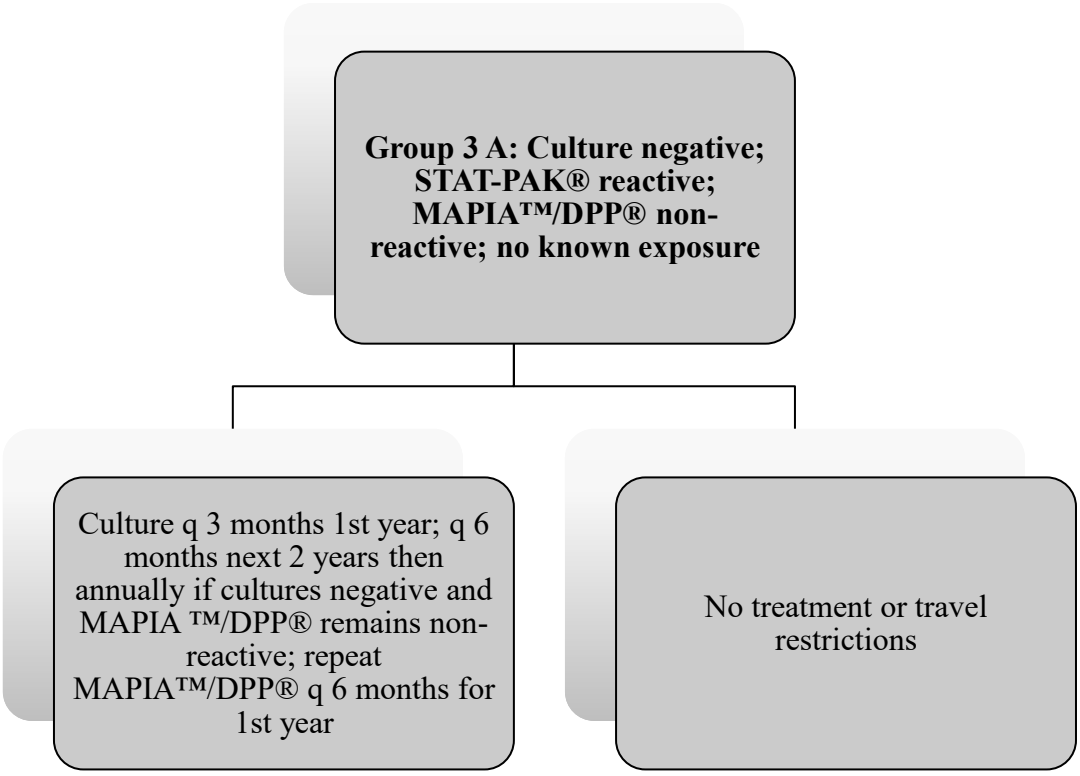
TB Management Group 1



TB Management Group 2



TB Management Group 3A



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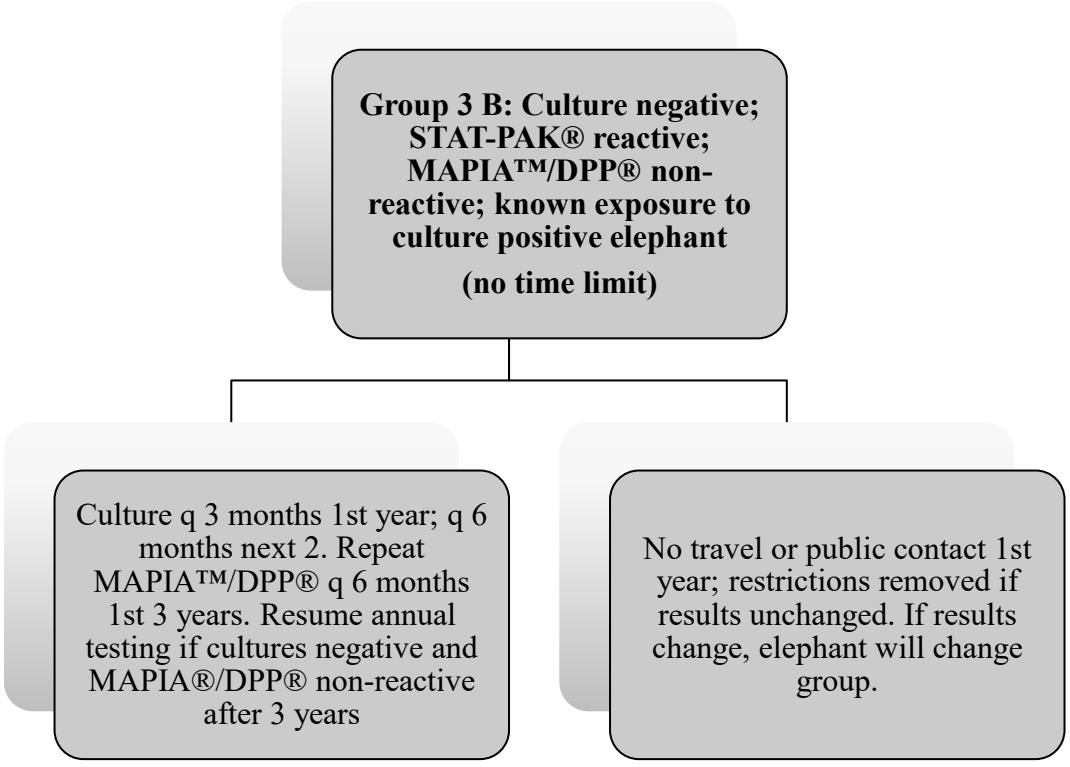

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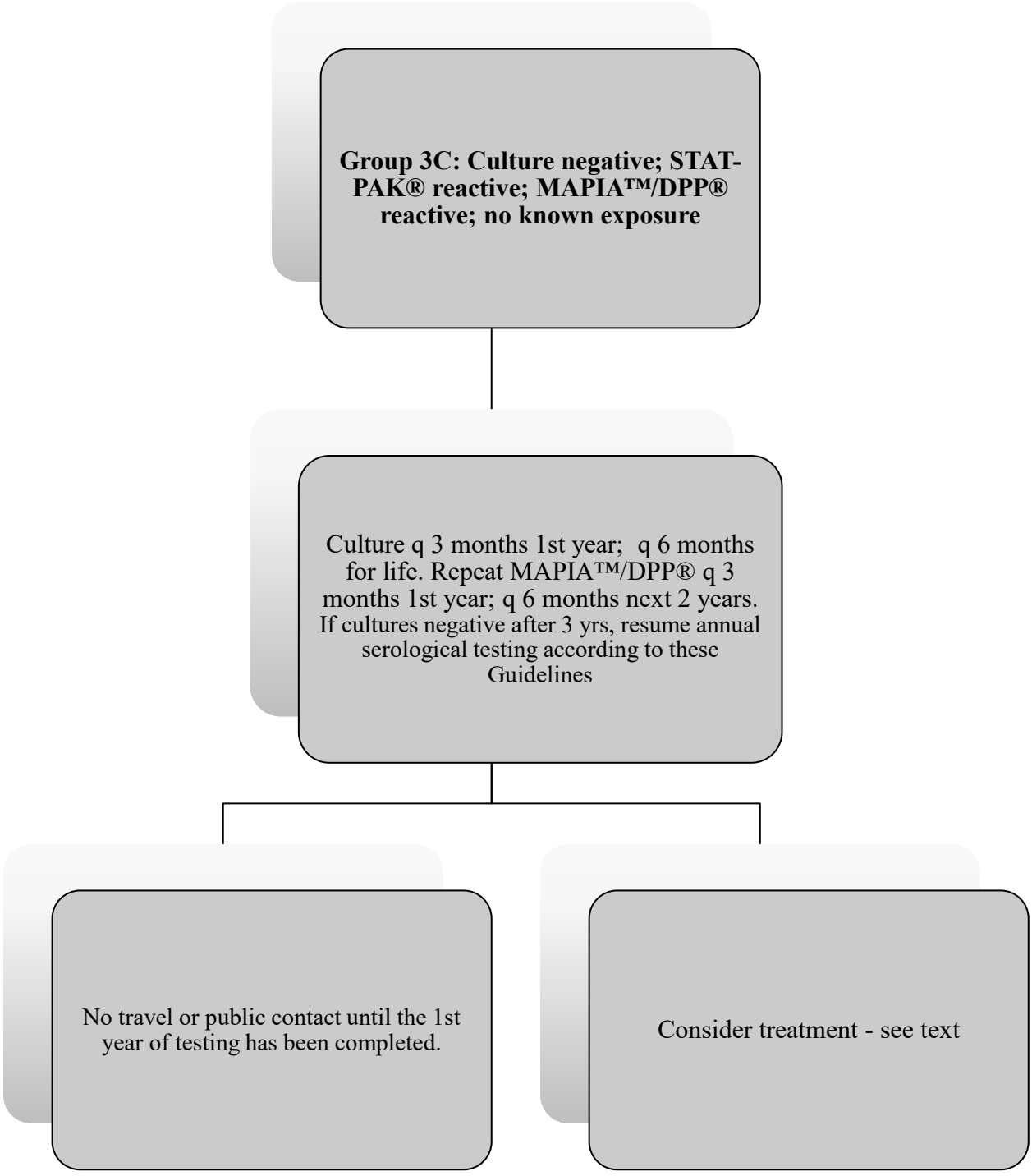
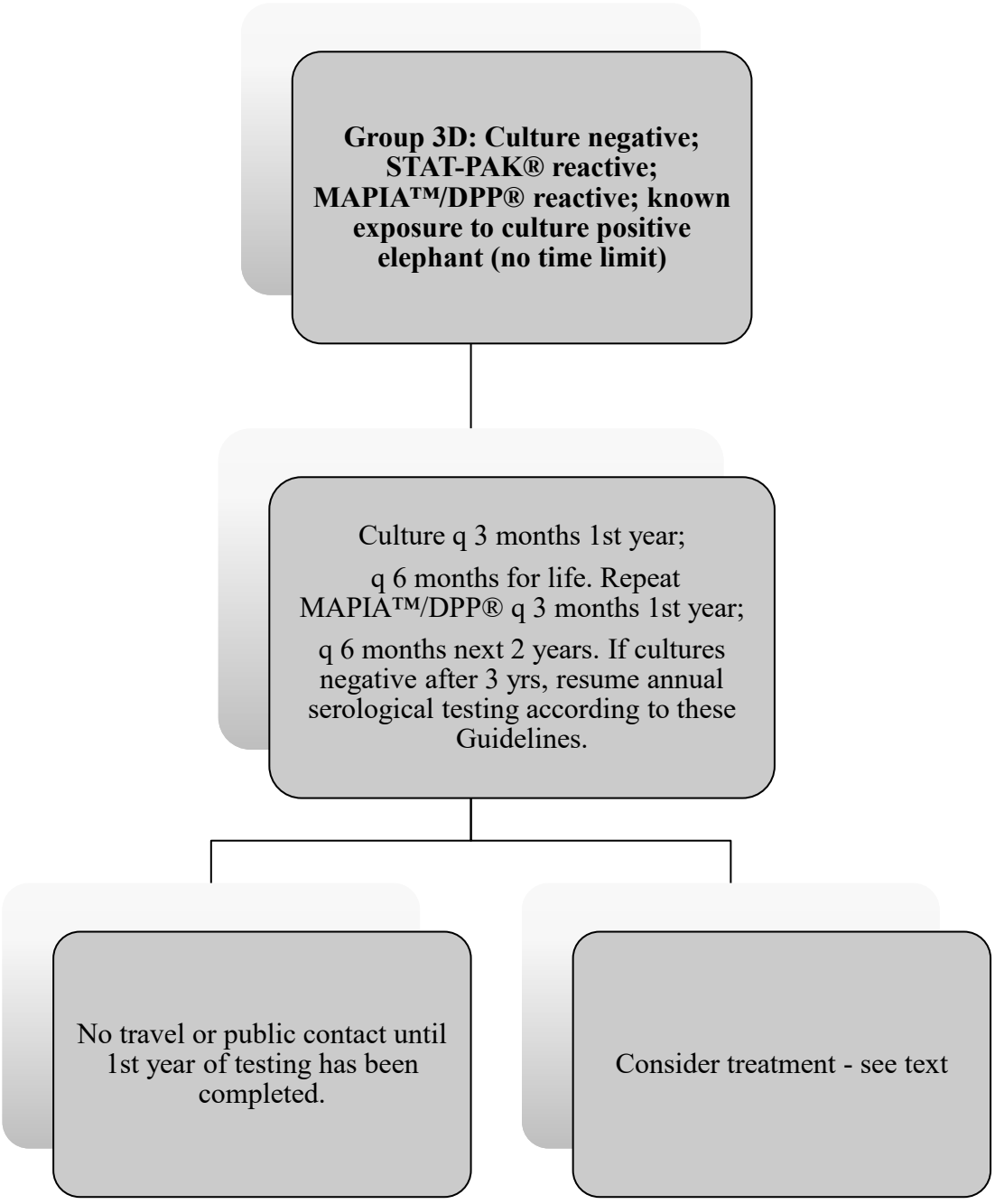


Management Group 3D



Management Group 4