Evaluation of DNA extraction techniques for detecting *Mycobacterium tuberculosis*-complex organisms in Asian elephant trunk washes

**Running title:** Mycobacteria DNA extraction techniques in elephants

**Authors:** Meagan K. Kay, University of California, Davis, School of Veterinary Medicine

Lyndsey Linke, Animal Population Health Institute, Colorado State University

Joni Triantis, Animal Population Health Institute, Colorado State University

M. D. Salman*, Animal Population Health Institute, Colorado State University

R. Scott Larsen, University of California, Davis, School of Veterinary Medicine

**Work performed at:** Animal Population Health Institute, College of Veterinary Medicine and Biomedical Sciences, Colorado State University, Fort Collins, CO 80523

**Corresponding Author:** Mailing address: M.D. Salman, Campus Stop 1644, Animal Population Health Institute, College of Veterinary Medicine and Biomedical Sciences, Colorado State University, Fort Collins, CO 80523-1644; Phone: 970-297-0353; Fax: 970-297-5228; Email: M.D.Salman@colostate.edu
ABSTRACT

Rapid and sensitive diagnostic assays for detection of tuberculous mycobacteria in elephants are lacking. DNA extraction and polymerase chain reaction (PCR) analysis is useful for tuberculosis screening in many species but has not been validated on elephant trunk wash samples. We estimated the analytical sensitivity and specificity of three DNA extraction methods to detect *Mycobacterium tuberculosis*-complex organisms in trunk wash specimens. A ZR Soil Microbe DNA Kit (ZR) and a traditional salt and ethanol precipitation (TSEP) approach were evaluated under three different treatment conditions: heat treatment, phenol treatment, and contamination with *M. avium*. A third approach, using a column filtration method, was evaluated for samples contaminated with soil. Trunk wash samples from uninfected elephants were spiked with varying concentrations of *M. bovis* cells and subjected to the described treatment conditions prior to DNA extraction. Extracted DNA was amplified using IS6110-targeted PCR analysis. The ZR and TSEP methods detected as low as 1–5 and 10 *M. bovis* cells per 1.5 ml trunk wash respectively, under all three conditions. Depending on the amount of soil present, the column filtration method detected as low as 5–50 *M. bovis* cells per 1.5 ml trunk wash. Analytical specificity was assessed by DNA extraction from species of non-tuberculous mycobacteria and amplification using the same PCR technique. Only *M. bovis* DNA was amplified, indicating 100% analytical specificity of this PCR technique. Our results indicate these DNA extraction techniques offer promise as useful tests for detection of *M. tuberculosis*-complex organisms in elephant trunk wash specimens.
INTRODUCTION

Tuberculosis (TB) is a highly contagious bacterial infection caused by organisms in the 
*Mycobacterium tuberculosis*-complex, most notably *Mycobacterium tuberculosis* or *M. bovis.*

*M. tuberculosis* typically affects humans and non-human primates, but has been found in many 
other species, including elephants (17, 19). Since 1996, TB has been diagnosed in many captive 
Asian elephants (*Elephas maximus*) housed in North America (14). Tuberculosis in elephants is 
typically caused by *M. tuberculosis*, although *M. bovis* is also reported to infect these species 
(10). Between 1994 and 2005, *M. tuberculosis* was detected in 31 Asian elephants and 3 
African elephants in captivity the United States (10). Based on estimates of approximately 535 
captive elephants in the U.S. (14), there is an estimated prevalence of 6.3%; however this 
estimate does not differentiate Asian elephants from African elephants. The disease also occurs 
in Asian elephants in Asia, and is likely due to both *M. tuberculosis* and *M. bovis* in Asian 
countries (1, 12). Research understanding the epidemiology of tuberculosis in elephants in this 
part of the world is still in its early stages. In recent studies, prevalence of TB infection in Asian 
elephants was estimated to be 13% in Nepal and 15% in India (1, 12), although it is not yet 
clear how many of these infections are due to *M. tuberculosis* and how many from *M. bovis.* 

Clinical signs of tuberculosis in elephants are variable. Some animals develop cavitatory 
lesions of the lungs and become debilitated, while many others lack clinical signs (14). Both 
clinically and sub-clinically affected animals have the potential to spread the disease to other 
elephants, and to humans, through trunk secretions or other bodily fluids (13). Attention has been 
directed at assessing the extent of TB among elephants in North America and improving 
diagnostic techniques that enable early identification of infected animals.
The number of Asian elephants is dropping worldwide due to habitat loss, poaching, and human conflict over resources (16). If left uncontrolled, a highly contagious disease such as TB could cause substantial morbidity and mortality in elephant herds, further contributing to the decline in elephant numbers. Furthermore, an outbreak of tuberculosis among elephants in a zoo or circus setting could put numerous other animals and people at risk of infection. Treatment of TB in elephants requires several months of costly medication and may impose financial hardships on elephant-owning institutions (20). Infected elephants may also pose a threat to human health, as noted by at least one case in which an elephant keeper and an elephant shared the same strain of *M. tuberculosis* (9). Thus, early diagnosis of tuberculosis is an essential step in effective management of the disease and is critical to reducing the number of new cases.

Early diagnosis of TB requires the use of screening tests that are accurate, easily implemented, and cost effective (14). Elephants typically lack clinical signs throughout most of the *M. tuberculosis* infection period, so reliance on clinical signs is an insensitive mechanism of detecting disease. Serological techniques have recently been found to be useful for determining infection status, but antibody titer may remain positive after treatment, and these tests do not definitively prove the presence of *M. tuberculosis*-complex organisms (6, 8). Currently, trunk wash culture serves as the “gold standard” for diagnosing tuberculosis in elephants (14, 20). This diagnostic technique, however, has low sensitivity, requiring >100 organisms/ml for detection, and can take up to eight weeks for the bacteria to grow in culture, during which time the bacteria may spread to other animals (11, 17, 18, 19). This long testing time interval also results in travel restrictions while samples are processed. These delays are problematic for circuses, which rely heavily on interstate travel, and for zoological institutions that are transferring animals for breeding and other management purposes. Culture samples are also
susceptible to overgrowth from non-tuberculous mycobacteria or other organisms that may result in false negative results (11). These, and other issues associated with culturing trunk wash samples, have prompted exploration of alternative mycobacterial detection methods for TB screening in elephants. Extraction of nucleic acids and subsequent polymerase chain reaction (PCR) analysis can be a rapid and sensitive diagnostic technique for detecting tuberculosis bacteria in tissues, soil, feces, and nasal swabs and could serve as a potentially useful alternative or complement to trunk wash culture for TB screening in elephants (7, 14). However, DNA extraction methods and PCR have not been fully evaluated or validated for elephant trunk washes (14). It is essential to estimate the analytical sensitivity and specificity of a DNA extraction technique, along with PCR analysis for diagnosis of TB from trunk washes.

This study aimed to estimate the analytical sensitivity and specificity of three extraction techniques for detection of *M. tuberculosis*-complex DNA from elephant trunk wash samples. To determine optimal testing parameters, and most appropriately design relevant extraction methods, several factors were considered, including specific handling methods during shipping, the presence of substances that are inhibitory to PCR (soil, grains, or grasses), and the presence of other mycobacterial species in a collected trunk wash sample. Considering the variability in resources and equipment availability among laboratories around the world, three different DNA extraction techniques were analyzed and compared under different sample treatment conditions. The presence of organic materials (e.g. grasses and soil) in trunk wash samples could be inhibitory to PCR, so the commercial ZR Soil Microbe DNA Kit (Zymo Research Corp., Orange, CA) was chosen due to past performance for detecting organisms in soil and feed samples (data not shown). Additionally, this kit contains specialized reagents formulated to breakdown polyphenols and humic acids that can be present in the soil. It includes a final filtration column
to remove trace amounts of inhibitory substances that are present in the eluted DNA. Another
technique that was evaluated was a traditional salt and ethanol precipitation (TSEP) approach,
utilizing non-commercial components. The third evaluated technique was a column filtration
method that uses a series of non-commercial buffers that are appropriate for extraction of DNA
in the presence of soil.

There are particular treatment requirements for shipment of biological samples depending
on their country of origin and infectious status. Many captive and free-ranging elephants live in
countries where FMD is endemic. As such, elephant samples shipped from those countries must
be treated prior to importation into the U.S. The approved methods for pre-importation treatment
of samples from these countries include treatment with: heat at 72°C for 30 minutes; a solution
with a pH of 5.5 or less for 30 minutes; a solution with a pH of 10 or greater for 2 hours; 10%
formalin; 0.2% glutaraldehyde; or 0.4% betapropriolactone. The method that is least likely to
affect PCR is heat inactivation, so the effect of this method of FMD treatment was analyzed in
this study.

In addition to international requirements for sample shipment, there are also domestic
requirements regarding shipment of samples of known disease status. If samples of known *M.
tuberculosis*-positive status are shipped within the U.S., standard regulations require treatment
with 5% phenol prior to shipping. Sample treatment with phenol was also performed in this
study in order to appropriately mimic clinical handling of infected samples shipped within the
United States.

Elephants spend a great deal of time manipulating soil and inhaling it into their trunks, so
the potential for contamination with soil mycobacteria is high. Consequently, there are concerns
about diagnostic sensitivity and interference from non-tuberculous mycobacteria that may be
present in trunk wash samples. To address these concerns, the effect of the presence of *M. avium* and other non-tuberculous mycobacteria in the described techniques were evaluated.

**MATERIALS AND METHODS**

**Study Subjects and Population**

Trunk wash samples were acquired from two adult female Asian elephants (*Elephas maximus*), living in a group with four other captive elephants in a zoological institution in California. Samples from these two elephants were presumed to be negative for infection with *M. tuberculosis*-complex organisms. Over the previous five years, a series of three trunk washes were collected annually from all of the elephants in the group and were submitted for mycobacterial culture testing at the National Veterinary Services Laboratory (Ames, IA). Culture results were consistently negative for *M. tuberculosis*-complex bacteria. This herd and institution had no previous history of TB and there had been no movement of new elephants into the herd within the past five years. Furthermore, all elephants had been tested repeatedly using a validated serologic test (6) with consistently negative results. All procedures performed on the elephants were approved by the UC Davis Animal Care and Use Committee (IACUC 12890).

**Trunk Wash Sampling**

In order to obtain a sufficient volume of trunk wash material for the development of the three extraction techniques, samples were obtained from both of these elephants during alternating weeks for a six-month period. Both elephants had been trained to allow the trunk wash procedure, which was routinely performed in order to meet U.S. Department of Agriculture (USDA) guidelines for control of tuberculosis in elephants (5). Approximately 60 ml of sterile saline was flushed into one nostril of the elephant’s trunk using a catheter tipped syringe. The
elephant was then instructed to raise the trunk and leave it elevated for 30 to 60 seconds, after which it lowered the trunk and exhaled. Trunk wash contents were collected in a sterile plastic bag and then aseptically transferred into a sterile, leak-proof, screw top container. Samples were stored at -80°C for two months, then thawed and 20% of each sample was pooled in order to create a representative and homogenous matrix for spiking trials. After mixing, the pool was aliquoted in 1.5 ml volumes into sterile 2.0 ml bead beater tubes (Sarstedt Inc., Newton, NC) and stored at -20°C. Each 1.5 ml trunk wash sample was later “spiked” with a defined quantity of *M. bovis* cells and each of the DNA extraction techniques described below utilized an entire spiked sample.

**Mycobacterial Cell Stocks**

In order to perform work at the Biosafety Level 2, cell stocks of killed *M. bovis* were used instead of *M. tuberculosis*. These two species of bacteria are both members of the *M. tuberculosis*-complex, are closely related, and have very similar antigenic presentation. The PCR technique used has been described to be specific for all members of the *M. tuberculosis*-complex (3). A cell stock of rinsed, killed *M. bovis* cells (strain 846146) was kindly provided by Dr. Ian Orme, Colorado State University. The cell stock was at a concentration of $5 \times 10^6$ colony forming units per ml, as determined by cell plate counts. On the day of each extraction, *M. bovis* cells were placed into a Beadbeater homogenizer (BioSpec Products, Inc., Bartlesville, OK) and subjected to mixing for 15 seconds at 3200 oscillations/minute in order to reduce cell clumping without breaking cells open. Cells were then diluted to reflect a theoretical “concentration” of 100, 50, 20, 10, 5, 1, and 0 cells per 50 µl of solution (TE Buffer with 0.02% Tween 80) for spiking into 1.5 ml negative trunk wash samples.
A cell stock of *M. avium* cells (subsp. *hominissuis* strain 2151) was kindly provided by Torsten Eckstein at Colorado State University. Cell plate counts were performed to quantify the *M. avium* stock, and the final concentration was determined to be $1 \times 10^9$ colony forming units per ml. On the day of each extraction, *M. avium* cells were placed into a Beadbeater homogenizer, as described above, for disruption of cell clumping.

**Preparation of Trunk Wash Spikes and Negative Extraction Controls**

Spiking with *M. bovis* was performed by thawing TB-negative 1.5 ml trunk wash aliquots to room temperature and using a positive displacement pipette to add 50 µl of the appropriate *M. bovis* dilution (100, 50, 20, 10, 5, or 1 cell). For each method of the investigation, a trunk wash sample spiked with 50 µl of the dilution buffer served as a negative extraction control (0 cells) to verify lack of cross contamination between extraction samples. In order to mimic typical handling of clinical specimens, in which samples are frozen after collection and shipped on ice, spiked and treated trunk washes were subsequently frozen at -70°C and thawed prior to continuing with the DNA extraction. After thawing, samples were centrifuged for 20 minutes at 11,000 × g (4°C). While carefully avoiding the cell pellet, the appropriate volume of trunk wash supernatant was removed to ensure that an exact volume of 150 µl remained for use in each of the extraction methods. Each trial was performed in triplicate. Detection limits for each extraction method and corresponding treatments were defined as the lowest concentration of mycobacteria detectable in all three replicate trials.

**Preparation of Trunk Wash - Phenol Treatment**

To mimic treatments that are required for U.S. domestic shipment of known MTB positive samples, the commercial ZR technique and the non-commercial TSEP technique were analyzed with spiked trunk wash samples that had been treated with a final concentration of 5% phenol.
phenol. Briefly, each 1.5 ml trunk wash sample was spiked as above, and 90 µl of 90% molecular grade phenol (5% final concentration) was added to each spiked trunk wash sample prior to freezing, centrifugation, and subsequent DNA extraction.

Preparation of Trunk Wash – Heat Treatment

The commercial ZR and the non-commercial TSEP techniques were analyzed with spiked trunk wash samples that had been subjected to heat treatment in order to mimic USDA treatment requirements for samples originating from countries endemic for foot and mouth disease (FMD). Briefly, each 1.5 ml trunk wash sample was spiked as above and then incubated for 30 minutes at 72°C prior to freezing, centrifugation and subsequent DNA extraction. Due to concerns about DNA degradation, samples subjected to the heat treatment technique were not treated with 5.0% phenol.

Preparation of Trunk Wash Containing both M. bovis and M. avium

To test the effect of the presence of large quantities of other mycobacteria on the performance of the commercial ZR and non-commercial TSEP techniques, copious amounts of M. avium were spiked concomitantly with M. bovis cells prior to extraction. Trunk wash samples were spiked with M. bovis as above, and then additionally spiked with 50 µl of M. avium cells to yield a final spiking concentration of $1 \times 10^8$ M. avium cells per 1.5 ml trunk wash sample. Phenol was added to a final concentration of 5% prior to freezing, centrifugation, and subsequent DNA extraction.

Preparation of Trunk Wash Containing Soil

To test the performance of all three extraction techniques in the presence of soil, known amounts of dirt from a presumed-negative elephant enclosure were added to trunk washes prior to extraction. Briefly, 300, 125, or 62.5 mg of soil was added to each 1.5 ml trunk wash sample,
prior to spiking with 100, 50, 20, 10, 5 or 0 cells of *M. bovis*. Centrifugation and subsequent DNA extraction were then performed.

**Commercial DNA Extraction Technique - ZR Soil Microbe DNA kit**

After completing the appropriate trunk wash preparation steps, samples were further purified and *M. bovis* DNA was extracted using the ZR Soil Microbe DNA Kit, according to the manufacturer’s instructions with slight modification. Briefly, beads from one ZR BashingBead™ Lysis Tube, along with 750 µl of the Zymo Soil Lysis Buffer solution, were added to each sample tube which already contained the remaining cell pellet and approximately 150 µl of supernatant that had been generated in the trunk wash preparation steps. Samples were then placed into a Beadbeater homogenizer and subjected to mechanical disruption with 2 pulses for 30 seconds at 3200 oscillations/minute. They were then incubated for 10 minutes at 100°C in order to ensure complete cellular lysis and cell inactivation. Due to concerns with DNA degradation, this final 100°C incubation was not performed on samples that had been prepared according to the phenol treatment method. Samples were centrifuged at 11,000 × g for 1 minute (room temperature) and 400 µl supernatant was transferred to one Zymo-Spin™ IV Spin Filter column. Samples were then treated as per manufacturer’s protocol, including filtration with the Zymo-Spin™ IV-HRC filter column. All DNA elutions were performed using 1.7 ml low-binding tubes (Axygen Scientific Inc., Union City, CA). Filtered DNA was then ready for IS6110 PCR testing. Resultant DNA preparations were diluted 1:2, 1:5, 1:10, and 1:20 in molecular grade water prior to PCR analysis to dilute potential inhibitors and excessive amounts of DNA.

**Non-Commercial DNA Extraction Technique – Traditional Salt/Ethanol Precipitation**
After completing the appropriate trunk wash preparation steps, samples were further purified and *M. bovis* DNA was extracted according to the TSEP procedure, as previously described with some modifications (1). Each sample tube contained the remaining cell pellet and approximately 150 µl of supernatant that had been generated in the trunk wash preparation steps. To this, fifteen 2.5 mm zirconia beads (BioSpec Products, Inc., Bartlesville, OK) and 0.5 g of 0.5 mm zirconia beads were added, as well as 250 µl of sterile salt homogenizing buffer (SHB, 0.64 M NaCl, 16 mM Tris-HCl pH 8.0, 3.2 mM EDTA pH 8.0). Samples were then subjected to mechanical disruption using a Beadbeater homogenizer with 2 pulses for 30 seconds at 3200 oscillations/minute. Following bead disruption, 40 µl of 20% sodium dodecyl sulfate and 14.85 µl of 20 mg/ml proteinase K (final concentration of 0.675 mg/ml) were added (Amresco Inc., Solon, OH). Samples were incubated at 65°C for 30 minutes, and then for 10 minutes at 100°C, in order to ensure complete cellular lysis and proteinase K inactivation. After incubation, samples were shaken vigorously ten times by hand and then placed on ice for five minutes to cool. Samples were then centrifuged for 10 minutes at 11,000 × g and 400 µl of supernatant was transferred to one 1.7 ml low binding tube containing 300 µl of 6 M NaCl (NaCl saturated H₂O). Samples were vortexed for 30 seconds at maximum speed prior to centrifugation for 20 minutes at 11,000 × g. Resulting supernatants were transferred to a new 1.7 ml low binding tube while carefully avoiding precipitated material. If particulate matter was inadvertently collected, an additional centrifugation step at 11,000 × g for 5 minutes was performed and the clarified supernatant transferred to a fresh low binding tube. An equal volume of cold 100% isopropanol was added, samples were thoroughly mixed via inversion or with a low speed vortexer, and then incubated at -70°C for 30 minutes (alternatively, samples may be stored overnight at -20°C prior to proceeding). Samples were centrifuged for 15 minutes at 11,000 × g (4°C) and washed twice.
Each wash consisted of the addition of 800 µl cold 70% ethanol, gentle re-suspension of the pellet, centrifugation for 5 minutes at 11,000 × g (4°C), and removal of the ethanol supernatant. After removal of the last wash, residual ethanol was evaporated off the DNA pellet by placing a Breathe-Easier® tube membrane (Diversified Biotech, Boston, MA) over each open tube and incubating at 65°C for 15 minutes or until DNA pellets were dry. Resulting pellets were rehydrated by the addition of 100 µl of TE buffer (Amresco Inc., Solon, OH) and incubated for 20 minutes at 72°C. Extracted DNA was then ready for IS6110 PCR testing and resultant DNA preparations were diluted 1:5, 1:10, 1:20, and 1:40 in molecular grade water prior to PCR analysis to dilute potential inhibitors and excessive amounts of DNA.

**Non-Commercial DNA Extraction Technique – Column Filtration**

After completing the appropriate trunk wash preparation steps (containing soil), samples were further purified and *M. bovis* DNA was extracted according to the following column filtration procedure utilizing buffer compositions based on recommendations provided by the column manufacturer (Epoch Biolabs, Inc, Sugar Land, TX). Each sample tube contained the remaining cell/soil pellet and approximately 150 µl of supernatant that had been generated in the trunk wash preparation steps. To this, fifteen 2.5 mm zirconia beads and 0.5 g of 0.5 mm zirconia beads were added, in addition to 250 µl of Lysis Buffer (40 mM Tris-HCl, 8 mM EDTA, ph 8.0 and 0.16 M NaOH). Samples were then subjected to mechanical disruption using a Beadbeater homogenizer with 2 pulses for 30 seconds at 3200 oscillations/minute. Following bead disruption, 40 µl of 20% sodium dodecyl sulfate and 14.85 µl of 20 mg/ml proteinase K (final concentration of 0.675 mg/ml) was added. Samples were incubated at 65°C for 30 minutes to activate the proteinase K and for 10 minutes at 100°C in order to ensure complete cellular lysis and proteinase K inactivation. After incubation, samples were shaken vigorously ten times by
hand and then placed on ice for five minutes to cool. Samples were then centrifuged for 5 minutes at 11,000 × g (room temperature), and supernatants were subjected to an additional centrifugation for further clarification. A total of 400 µl of clarified supernatant was transferred to one 1.7 ml low binding tube containing 400 µl of Binding Buffer (4 M Guanidine HCl, 0.5 M Potassium Acetate, pH 4.2) and samples were vortexed for 30 seconds at maximum speed prior to centrifugation for 10 minutes at 10,000 × g (room temperature). Resulting supernatants were transferred to one EconoSpin filter tube with attached lid (Epoch Biolabs, Inc, Sugar Land, TX) and centrifuged at 6,100 × g for 1 minute and 15 seconds (room temperature). The filtrate was discarded and the filter was transferred to a fresh collection tube. Five hundred µl of Wash ONE Buffer (5 M Guanidine HCl, 20 mM Tris-HCl, pH 6.6 in final concentration of 38% ETOH) was added to the filter column and the filter/collection tube was centrifuged at 6,100 × g for 1 minute and 15 seconds. A final rinse was conducted using 750 µl of Complete Wash TWO Buffer (20 mM NaCl, 2 mM Tris-HCl, pH 7.5, and 80% ETOH) and the samples were again centrifuged. The filtrate was discarded and the filter columns were centrifuged for an additional 1 minute at 11,000 × g to remove any residual ethanol. The EconoSpin filter column was transferred to a 1.7 ml low binding tube, 100 µl of warm Elution Buffer (10 mM Tris-HCl, pH 8.5) was added, and following incubation for 5 minutes at room temperature, samples were centrifuged at 6,100 × g for 1 minute and 15 seconds to elute the DNA. Extracted DNA was then ready for IS6110 PCR testing and resultant DNA preparations were diluted 1:10, 1:20, 1:40, and 1:60 in molecular grade water prior to PCR analysis to dilute potential inhibitors and excessive amounts of DNA.

Conventional PCR for Amplification of the IS6110 Insertion Sequence of the M. Tuberculosis Complex
Extracted DNA was amplified using conventional PCR targeting a 123 base pair segment of the IS6110 insertion sequence as previously described, with modifications (2, 3, 12). Briefly, each 25 µl reaction volume consisted of 1X Amplitaq Gold Buffer® II and 1.5 mM MgCl₂ (Applied Biosystems, Foster City, CA), 0.2 mM each nucleotide (Roche Applied Sciences, Indianapolis, IN), 0.4 µM each primer (forward primer sequence: CCGCGTAGGCGTCGG; reverse primer sequence: CCGCTCGTCCAGCGCCGGTTCGG), 1.875 U Amplitaq Gold® polymerase (Applied Biosystems, Foster City, CA), and 10 µl DNA. Each reaction was overlaid with 30 µl Chill Out® wax (Bio-Rad, Hercules, CA) to prevent evaporation and placed into an MJ Research 60 place thermal cycler (Bio-Rad, Hercules, CA). Thermal cycling conditions consisted of an initial incubation at 94°C for 10 minutes to activate the polymerase, followed by 51 cycles of 94°C for 45 seconds and 72°C for 2 minutes and 15 seconds, with the last 72°C incubation being extended to 10 minutes. PCR product was analyzed by agarose gel electrophoresis using the FlashGel® DNA System (Lonza Group Ltd, Basel Switzerland). Amplified products were visualized by UV light transillumination. Molecular weight markers (Lonza Group Ltd, Basel Switzerland) were concordantly run on the gels to aid in the calculation of the size of the amplified DNA fragments. Samples producing an expected band size of 123 base pairs were considered positive, and bands of any other size were considered negative. Electrophoretic results were scored in a blinded fashion by technicians with no knowledge of the concentration of mycobacteria or treatment type.

Testing With Non-Tuberculous Mycobacteria and Soil Microbes

DNA was extracted from cultures of multiple species of non-tuberculous mycobacteria and other soil microbes, including M. abscessus (ATCC 19977), M. avium (hominissuis, Str 2151), M.
avium paratuberculosis (ATCC 19698), M. chelonae (ATCC 35752), M. fortuitum (ATCC 19542), M. intracellulare (ATCC 13950), M. kansasii (ATCC 12478), M. marinum (ATCC 927), M. phlei (ATCC 11758), M. simiae (ATCC 25273), M. smegmatis (ATCC 23011), M. szulgai (ATCC 35799), M. terrae (ATCC 15755), and Nocardia asteroides (ATCC 3308). DNA was quantified using fluorometry and 10 pg of DNA of each of the species was tested in the IS6110-targeted PCR protocol. As above, the laboratory technician who interpreted the electrophoretic results was blinded to the species of Mycobacteria present in each well.

Data Analysis

The detection limit, or analytical sensitivity, for the IS6110-targeted PCR after using a particular extracting technique was reported as the lowest concentration of M. bovis cells that was detectable using this method in 3 out of 3 trials. The analytical specificity was reported as the ability of the assay to produce a 123-base pair product only when spiked with M. bovis and not in any of the negative controls or when spiked with non-tuberculous bacteria. Both analytical sensitivity and specificity estimates were reported as absolute figures rather than as proportions. Thus, no statistical inferences were performed on these estimates.

RESULTS

The lowest detection levels at which all three replicates spiked with M. bovis produced a 123-base pair product following DNA extraction and under each treatment condition (5% phenol, heat inactivation, and concomitant spiking with M. avium and M. bovis) are shown in Table 1. The optimal DNA dilution for the ZR method was 1:2 and the optimal dilution for the TSEP method was 1:10. Final dilutions were not expected to be uniform across all extraction methods due to variation in the procedures. Therefore, optimal DNA dilutions prior to PCR...
amplification were determined from the serial dilutions tested with each set of three replicate
spikes.

The non-commercial column filtration method was only analyzed in the presence of soil
and was not evaluated using any of the three treatment conditions indicated above. A
representation of 1.5 ml trunk wash with 300, 125 or 62.5 mg of soil is depicted in Figure 1. The
lowest detection levels at which all three replicates produced a 123-base pair product when
spiked with *M. bovis*, and containing each concentration of soil, are shown in Table 2. The
optimal DNA dilution for the non-commercial column filtration method was 1:20. Prior to this
study, the ZR method had routinely demonstrated the ability to detect a concentration of 1.25 *M.
bovis* cells per 500 mg of soil with DNA diluted at 1:5 prior to PCR analysis (data not shown).
For the purpose of comparison and to serve as a positive extraction control, the ZR method was
re-evaluated with 1.5 ml trunk wash containing 500 mg soil, spiked with 20, 10 and 5 *M. bovis*
cells. The detection limit of the TSEP method was greatly compromised in the presence of 62.5
mg of soil, so that method was not further evaluated in the soil detection limit analysis.

The analytical specificity of the IS6110-targeted PCR was evaluated after amplification
of DNA extracted from cultures of multiple species of non-tuberculous bacteria and other soil
microbes (*M. abscessus, M. avium hominissuis, M. chelonae, M. fortuitum, M. intracellulare, M.
marinum, M. avium paratuberculosis, M. phlei, M. simiae, M. smegmatis, M. szulgai, M. terrae,
M. kansasii and Nocardia asteroidis*). Only DNA originating from *M. bovis* cells was
amplifiable, resulting in a 123-bp band when analyzed by agarose gel electrophoresis. There
was no DNA amplification from any of the thirteen non-tuberculous mycobacterial cultures, nor
*Nocardia*, that were tested.
DISCUSSION

Results of this study suggest that DNA extraction and subsequent PCR analysis may provide a more rapid and sensitive alternative to mycobacterial culture for detection of *M. tuberculosis* or *M. bovis* in elephant trunk washes. The methods appear to be specific for *M. tuberculosis*-complex organisms because the IS6110 target sequence was amplified in samples spiked with *M. bovis*, but not in samples spiked with *M. avium* or other non-tuberculous mycobacteria. Using the extraction techniques outlined in this study, samples could be processed and results obtained in as little as two days after clinical submission, while providing a more cost-effective diagnostic assay compared to many other currently available techniques, including culture. These techniques are not labor intensive and can easily be performed in most clinical laboratories, minimizing the need to ship samples to regulated facilities for mycobacterial culture. Table 3 serves as a reference guide and an application summary for each DNA extraction technique. Briefly, this table demonstrates the robust nature of the ZR and TSEP techniques and their utility for detecting organisms on treated trunk wash samples. This will facilitate testing of samples from infected elephants and on samples that need to be imported from countries that are not classified as “FMD free”. Furthermore, the presence of large quantities of *M. avium* did not affect the detection limits of the ZR or the TSEP extraction method. The non-commercial column filtration method appears to be an excellent alternative to the commercial ZR method for processing trunk wash samples heavily saturated with amounts of contaminating soil. Results in Table 2 indicate that the analytical sensitivity of the column filtration technique is reduced as the amount of spiked soil is increased; however, even in the presence of 300 mg of soil, the column filtration technique routinely detects 50 *M. bovis* cells per 1.5 ml trunk wash. This method therefore provides a viable non-commercial alternative capable
of circumventing the inhibitory effects soil is known to elicit with PCR amplification.

Unfortunately, due to logistical constraints, it was not possible to evaluate the column filtration technique under conditions of phenol treatment, heat treatment, or contamination with *M. avium*.

In a previous study that compared mycobacterial culture with IS6110-targeted PCR of DNA extracted from infected human sputum samples, the DNA extraction and PCR amplification technique detected the presence of *M. tuberculosis* in several samples that were negative on mycobacterial culture (4). From a clinical standpoint, these findings indicate that molecular techniques may be more sensitive than culture for detecting MTB complex and/or detecting DNA from dead or non-culturable cells. Infected elephants shed *M. tuberculosis*-complex bacteria intermittently throughout their infection, which is a contributing factor to the low sensitivity of mycobacterial culture. This intermittent shedding could potentially affect the sensitivity of DNA extraction from clinical samples as well. Initial results of our study suggest that these extraction techniques are highly sensitive and capable of detecting *M. bovis* cells present in very low concentrations (18).

Currently, one of the issues associated with developing new techniques for diagnosing tuberculosis in elephants is the lack of a gold standard test, and using mycobacterial culture as the gold standard can pose several problems. Based on a number of strict criteria, the samples used in this study were presumed negative. However, there is currently no diagnostic technique for trunk wash samples that can definitively classify an animal as uninfected, allowing the potential for false negative samples to be used in the study. Therefore, if any of the culture negative samples acquired had contained *M. tuberculosis*-complex bacteria, the analytical sensitivities of these extraction techniques would overestimate the true detection limit of each method. Although use of a validated negative control was not feasible, the medical history,
husbandry status, and historic TB test results suggest that false-negative status is unlikely. The inclusion of elephant trunk wash extraction controls, spiked with 0 cells, provided a means for assessing any potential cross-contamination between samples during the extraction procedures as well as validation of the negative status of the samples utilized in these studies. Under no circumstances was there a negative extraction control sample that yielded an amplifiable 123-base pair band.

This study serves as a starting point for validating DNA extraction techniques from elephant trunk wash samples under a variety of conditions. While it does not validate the techniques using clinical samples from infected animals, it is hoped that the techniques can be used for future evaluation of TB-infection status in elephants. It would be useful to obtain clinical specimens from infected elephants and compare DNA extraction and PCR to mycobacterial culture and serology. While ideal, this task is somewhat difficult to achieve due to the intermittent detection of tuberculosis shedding in captive elephants and the wide geographic distribution of elephants throughout North America. These techniques provide promise for detecting M. tuberculosis-complex in Asian elephants; however, when determining infection status, PCR should not be used alone, but should be part of a battery of tests including culture and serology.

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REFERENCES


Table 1. Detection limits using the ZR and TSEP extraction techniques.

<table>
<thead>
<tr>
<th>Treatment Condition</th>
<th>M. bovis cells</th>
<th>ZR</th>
<th>TSEP</th>
</tr>
</thead>
<tbody>
<tr>
<td>5% Phenol</td>
<td>5</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Heat Treatment (72 °C)</td>
<td>1</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>M. avium spike</td>
<td>1</td>
<td>10</td>
<td></td>
</tr>
</tbody>
</table>

*a* Based on three replicate DNA extractions using the indicated treatment condition.

*b* Lowest concentration of *M. bovis* cells detected per 1.5 ml sample.

Table 2. Detection limits using the column filtration technique.

<table>
<thead>
<tr>
<th>Soil (milligrams)</th>
<th>M. bovis cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>300</td>
<td>50</td>
</tr>
<tr>
<td>125</td>
<td>20</td>
</tr>
<tr>
<td>62.5</td>
<td>5</td>
</tr>
</tbody>
</table>

*a* Based on three replicate DNA extractions at the indicated soil concentration.

*b* Concentration of soil spiked into each 1.5 ml trunk wash sample.

*c* Lowest concentration of *M. bovis* cells detected.
### Table 3. Reference guide and application summary for each DNA extraction technique evaluated

<table>
<thead>
<tr>
<th>Technique</th>
<th>Cost Per Sample ($)</th>
<th>Process Timeistica</th>
<th>Soil Tolerantb</th>
<th>Phenol Tolerant</th>
<th>Heat Tolerant</th>
<th>Analytical Sensitivityc</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZR</td>
<td>4.20</td>
<td>2</td>
<td>500</td>
<td>yes</td>
<td>yes</td>
<td>1.25 5 1 1</td>
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<tr>
<td>TSEP</td>
<td>1.40</td>
<td>4</td>
<td>&lt; 62.5</td>
<td>yes</td>
<td>yes</td>
<td>*NT 10 10 10</td>
</tr>
<tr>
<td>Column Filtration</td>
<td>2.40</td>
<td>3.3</td>
<td>300</td>
<td>*NT</td>
<td>*NT</td>
<td>50 *NT *NT *NT</td>
</tr>
</tbody>
</table>

*a Time in hours based on 20 samples

*b Maximum soil (mg per 1.5 ml sample) allowable without inhibiting TB DNA detection

*c Lowest concentration of *M. bovis* cells (# cells per 1.5 ml sample) detected

*d Based on maximum soil tolerance concentrations

*e When spiked with copious amounts of *M. avium*

*NT = not tested

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Figure 1. After allowing the soil to settle in a 2.0 ml tube, this picture represents a 1.5 ml trunk wash sample with 300 mg soil (a), 125 mg soil (b), 62.5 mg soil (c), and a normally acquired trunk wash sample with minimal soil contamination (d).