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Published Ahead of Print 15 December 2010.

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Evaluation of DNA Extraction Techniques for Detecting *Mycobacterium tuberculosis* Complex Organisms in Asian Elephant Trunk Wash Samples

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Received 21 April 2010/Returned for modification 26 October 2010/Accepted 3 December 2010

Rapid and sensitive diagnostic assays for the detection of tuberculous mycobacteria in elephants are lacking. DNA extraction with 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 *Mycobacterium 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 various 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 to 5 *M. bovis* cells and 10 *M. bovis* cells, respectively, per 1.5 ml of trunk wash under all three conditions. Depending on the amount of soil present, the column filtration method detected as low as 5 to 50 *M. bovis* cells per 1.5 ml of trunk wash. Analytical specificity was assessed by DNA extraction from species of nontuberculous 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 that these DNA extraction techniques offer promise as useful tests for detection of *M. tuberculosis* complex organisms in elephant trunk wash specimens.

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 nonhuman primates but has also 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). TB 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 in the United States (10). Based on estimates of approximately 535 captive elephants in the United States (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 investigating the epidemiology of TB 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 are due to *M. bovis*.

Clinical signs of TB in elephants are variable. Some animals develop cavitory lesions of the lungs and become debilitated, while many others lack clinical signs (14). Both clinically and subclinically 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 competition for 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 TB 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 TB 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 \textit{M. tuberculosis} complex organisms \cite{6,8}. Currently, trunk wash culture serves as the “gold standard” for diagnosing TB in elephants \cite{14,20}. This diagnostic technique, however, has low sensitivity, requiring \(>100\) organisms/ml for detection, and can take up to 8 weeks for the bacteria to grow in culture, during which time the bacteria may spread to other animals \cite{11,17–19}. This long testing time interval also results in travel restrictions while the 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 nontuberculous mycobacteria or other organisms that may result in false-negative results \cite{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 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 \cite{7,14}. However, DNA extraction methods and PCR have not been fully evaluated or validated for elephant trunk wash samples have prompted exploration of alternative mycobacterial detection methods for TB screening in elephants. Extraction of nucleic acids and subsequent 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 \cite{7,14}. However, DNA extraction methods and PCR have not been fully evaluated or validated for elephant trunk wash samples. To address these concerns, the effect of the presence of \textit{M. avium} and other nontuberculous mycobacteria in the described techniques were evaluated.

\textbf{MATERIALS AND METHODS}

\textbf{Study subjects and population.} Trunk wash samples were acquired from two adult female Asian elephants (\textit{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 \textit{M. tuberculosis} complex organisms. Over the previous 5 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 \textit{M. tuberculosis} complex bacteria. This herd and institution had no history of TB, and there had been no movement of new elephants into the herd within the past 5 years. Furthermore, all elephants had been tested repeatedly using a validated serologic test \cite{6} with consistently negative results. All procedures performed on the elephants were approved by the UC Davis Animal Care and Use Committee (IACUC 12890).

\textbf{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 6-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 \cite{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 s, 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 2 months and 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 divided into aliquots 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 \textit{M. bovis} cells, and each of the DNA extraction techniques described below utilized an entire spiked sample.

\textbf{Mycobacterial cell stocks.} In order to perform work at biosafety level 2, cell stocks of \textit{M. bovis} were used instead of \textit{M. tuberculosis}. These two species of bacteria are both members of the \textit{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 \textit{M. tuberculosis} complex \cite{3}. A cell stock of rinsed, killed \textit{M. bovis} cells (strain 846146) was kindly provided by Ian...
On the day of each extraction, the samples were centrifuged for 20 min at 11,000 \( \times g \) (4°C). After centrifugation, the supernatants were transferred to one new 1.7-ml low binding tube (EconoSpin filter tube with attached lid (Epoch Biolabs) and centrifuged at 10,000 \( \times g \) (room temperature). The resulting DNA preparations were diluted 1:5, 1:10, 1:20, and 1:40 in molecular-grade water. Resultant DNA preparations were subjected to mechanical disruption using a BeadBeater homogenizer with two pulses for 30 s at 3,200 oscillations/min. After bead disruption, 40 \( \mu l \) of 20% sodium dodecyl sulfate and 14.85 \( \mu l \) of 20 mg of proteinase K/ml (final concentration, 0.675 mg/ml) were added (Amresco, Inc., Solon, OH). Samples were incubated at 65°C for 30 min and then for 10 min at 100°C in order to ensure complete cellular lysis and proteinase K inactivation. After incubation, the samples were shaken vigorously 10 times by hand and then placed on ice for 5 min to cool. Samples were then centrifuged for 10 min at 11,000 \( \times g \), and 400 \( \mu l \) of supernatant were transferred to a new 1.7-ml low binding tube containing 300 \( \mu l \) of 6 M NaCl (NaCl saturation buffer) for 15 min at 4°C (alternatively, samples may be stored overnight at −20°C prior to proceeding). Samples were centrifuged for 15 min at 11,000 \( \times g \) and washed twice. Each wash consisted of the addition of 800 \( \mu l \) of cold 70% ethanol, gentle resuspension of the pellet, centrifugation for 5 min at 11,000 \( \times g \), 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 Bio-tech, Boston, MA) over each open tube and incubating at 65°C for 15 min or until DNA pellets were dry. The resulting pellets were rehydrated by the addition of 100 \( \mu l \) of Tris-EDTA buffer (Ambrosec, Inc., Solon, OH) and incubated for 20 min 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.

Commercial DNA extraction technique: traditional salt-ethanol precipitation. After completion of the appropriate trunk wash preparation steps, 900 \( \mu l \) of supernatant was transferred to one Zymo-Spin IV spin 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.

Noncommercial DNA extraction technique: traditional salt-ethanol precipitation. After completion of the appropriate trunk wash preparation steps, the 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 \( \sim 150 \) \( \mu l \) of supernatant that had been generated in the trunk wash preparation steps. To this, 15.25-mm zirconia beads (BioSpec) and 0.5 g of 0.5-mm zirconia beads were added, as well as 250 \( \mu l \) 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 two pulses for 30 s at 3,200 oscillations/min. After bead disruption, 40 \( \mu l \) of 20% sodium dodecyl sulfate and 14.85 \( \mu l \) of 20 mg of proteinase K/ml (final concentration, 0.675 mg/ml) were added (Amresco, Inc., Solon, OH). Samples were incubated at 65°C for 30 min and then for 10 min at 100°C in order to ensure complete cellular lysis and proteinase K inactivation. After incubation, the samples were shaken vigorously 10 times by hand and then placed on ice for 5 min to cool. Samples were then centrifuged for 10 min at 11,000 \( \times g \), and 400 \( \mu l \) of supernatant was transferred to one 1.7-ml low binding tube containing 300 \( \mu l \) of 6 M NaCl (NaCl saturation buffer) for 15 min at 4°C (alternatively, samples may be stored overnight at −20°C prior to proceeding). Samples were centrifuged for 15 min at 11,000 \( \times g \) and washed twice. Each wash consisted of the addition of 800 \( \mu l \) of cold 70% ethanol, gentle resuspension of the pellet, centrifugation for 5 min at 11,000 \( \times g \), 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 Bio-tech, Boston, MA) over each open tube and incubating at 65°C for 15 min or until DNA pellets were dry. The resulting pellets were rehydrated by the addition of 100 \( \mu l \) of Tris-EDTA buffer (Ambrosec, Inc., Solon, OH) and incubated for 20 min 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.

Commercial DNA extraction technique-ZR soil microbe DNA kit. To mimic treatments that are required for U.S. domestic shipment of known \( M. \) tuberculosis-positive samples, the commercial ZR technique and the noncommercial TSEP technique were analyzed with spiked trunk wash samples that had been treated with a final concentration of 100 \( \times 10^5 \) cells per 1.5-ml trunk wash sample. Phenol was as described above, and 90 \% 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 containing both \( M. \) bovis and \( M. \) avium. To test the effects of preparing large quantities of other mycobacteria on the performance of the commercial ZR and noncommercial 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 described above and then additionally spiked with 50 \( \mu l \) of \( M. \) avium cells to yield a final spiking concentration of 10 \( \times 10^5 \) \( M. \) 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 65.2 mg of soil was added to each 1.5-ml trunk wash sample, prior to spiking with 100, 50, 20, 10, 5, or 0 \( \mu l \) of \( M. \) bovis. Centrifugation and subsequent DNA extraction were then performed.

Commercial DNA extraction technique-ZR soil microbe DNA kit. After completion of the appropriate trunk wash preparation steps, the 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., Sugarland, TX). Each sample tube contained the remaining cell-soil pellet and ca. 150 \( \mu l \) of supernatant that had been generated in the trunk wash preparation steps. To this, 15.25-mm zirconia beads and 0.5 g of 0.5-mm zirconia beads were added, in addition to 250 \( \mu l \) of lysis buffer (40 mM Tris-HCl, 8 mM EDTA [pH 8.0], 0.16 M NaOH). Samples were then subjected to mechanical disruption using a BeadBeater homogenizer with 2 pulses for 30 s at 3,200 oscillations/min. After bead disruption, 40 \( \mu l \) of 20% sodium dodecyl sulfate and 14.85 \( \mu l \) of 20 mg of proteinase K/ml (final concentration, 0.675 mg/ml) were added. Samples were then incubated at 65°C for 30 min and then for 10 min at 100°C in order to ensure complete cellular lysis and proteinase K inactivation. After incubation, the samples were shaken vigorously 10 times by hand and then placed on ice for 5 min to cool. Samples were then centrifuged for 5 min at 11,000 \( \times g \) (room temperature), and supernatants were subjected to an additional centrifugation for further clarification. A total of 400 \( \mu l \) of clarified supernatant was transferred to one 1.7-ml low binding tube containing 400 \( \mu l \) of binding buffer (4 M Guanidine HCl, 0.5 M Tris-HCl, pH 4.2) and 400 \( \mu l \) of 70% ethanol were added. Samples were incubated at 65°C for 30 min to activate the proteinase K and for 10 min at 100°C in order to ensure complete cellular lysis and proteinase K inactivation. After incubation, samples were shaken vigorously 10 times by hand and then placed on ice for 5 min to cool. Samples were then centrifuged for 5 min at 11,000 \( \times g \) (room temperature), and supernatants were subjected to an additional centrifugation for further clarification. A total of 400 \( \mu l \) of clarified supernatant was transferred to one 1.7-ml low binding tube containing 400 \( \mu l \) of binding buffer (4 M Guanidine HCl, 0.5 M Tris-HCl, pH 4.2) and 400 \( \mu l \) of 70% ethanol were added. Samples were then incubated at 65°C for 30 min to activate the proteinase K and for 10 min at 100°C in order to ensure complete cellular lysis and proteinase K inactivation. After incubation, the samples were then centrifuged for 10 min at 10,000 \( \times g \) (room temperature). The resulting supernatants were transferred to one EconoSpin filter tube with attached lid (Epoch Biologics) and centrifuged at 6,100 \( \times g \) for 1 min and 15 s (room temperature). The filtrate was discarded, and
bacterial species present in each well. The technician who interpreted the electrophoretic results was blinded to the mycobacterial species. All work was performed at the Animal Population Health Institute, College of Veterinary Medicine and Biomedical Sciences, Colorado State University, Fort Collins, CO.

RESULTS

The lowest detection levels at which all three replicates spiked with M. bovis produced a 123-bp product after 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 noncommercial 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 of trunk wash with 300, 125, or 62.5 mg of soil is depicted in Fig. 1. The lowest detection levels at which all three replicates produced a 123-bp product when spiked with M. bovis, and containing each concentration of soil, are shown in Table 2. The optimal DNA dilution for the noncommercial column filtration method was 1:20. Prior to the present 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 reevaluated with 1.5 ml of trunk wash containing 500 mg of soil, spiked with M. bovis.

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 three trials. The analytical specificity was reported as the ability of the assay to produce a 123-bp product only when spiked with M. bovis and not in any of the negative controls or when spiked with nontuberculous 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.

### TABLE 1. Detection limits using the ZR and TSEP extraction techniques

<table>
<thead>
<tr>
<th>Treatment condition</th>
<th>No. of M. bovis cells&lt;sup&gt;a&lt;/sup&gt;</th>
<th>ZR</th>
<th>TSEP</th>
</tr>
</thead>
<tbody>
<tr>
<td>5% Phenol</td>
<td>5</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Heat treatment (72°C)</td>
<td>1</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>M. avium spike</td>
<td>1</td>
<td>10</td>
<td>10</td>
</tr>
</tbody>
</table>

<sup>a</sup> Based on three replicate DNA extractions using the indicated treatment condition.

<sup>b</sup> Values represent the 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 sample (mg)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>No. of M. bovis cells&lt;sup&gt;c&lt;/sup&gt;</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>

<sup>a</sup> Based on three replicate DNA extractions at the indicated soil concentration.

<sup>b</sup> Concentration of soil spiked into each 1.5-ml trunk wash sample.

<sup>c</sup> Values represent the lowest concentration of M. bovis cells detected.
saturated with amounts of contaminating soil. The 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 of trunk wash. This method therefore provides a viable noncommercial 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 M. tuberculosis complex and/or detecting DNA from dead or nonculturable 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 the present 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 be overestimated. Although the 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 a 123-bp band.

This study serves as a starting point for validating DNA extraction techniques from elephant trunk wash samples under a variety of conditions. Although 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 rather should be part of a battery of tests, including culture and serology.

ACKNOWLEDGMENTS

This study was supported by a Wildlife Health Center Fellowship and the STAR program, School of Veterinary Medicine, University of California, Davis, CA, and from the Colorado State University-Program of Economically Infectious Animal Diseases through a special grant from USDA/NIFA.

We thank the animal care staff and veterinarians at the zoological institution that contributed samples to this investigation.

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18. Reference deleted.