Journal of Clinical Microbiology

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J. Clin. Microbiol. **2011, 49(2):618. DOI: 10.1128/JCM.00807-10.**
Published Ahead of Print 15 December 2010.

JOURNAL OF CLINICAL MICROBIOLOGY, Feb. 2011, p. 618–623 Vol. 49, No. 2
0095-1137/11/$12.00 doi:10.1128/JCM.00807-10

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 IS*6110*-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 cavitary 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

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▽ Published ahead of print on 15 December 2010.

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 TB in elephants (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 (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 (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 (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). In addition, 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 noncommercial components. The third evaluated technique was a column filtration method that uses a series of noncommercial buffers that are appropriate for the extraction of DNA in the presence of soil.

There are particular treatment requirements for the shipment of biological samples depending on their country of origin and infectious status. Many captive and free-ranging elephants live in countries where foot and mouth disease (FMD) is endemic. As such, elephant samples shipped from those countries must be treated prior to importation into the United States. The approved methods for preimportation treatment of samples from these countries include treatment with the following: heat at 72°C for 30 min, a solution with a pH of 5.5 or less for 30 min, a solution with a pH of ≥10 for 2 h, 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 here.

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 United States, standard regulations require treatment with 5% phenol prior to shipping. Sample treatment with phenol was also performed in the present 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 nontuberculous mycobacteria that may be present in trunk wash samples. To address these concerns, the effect of the presence of *M. avium* and other nontuberculous 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 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 *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 (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 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 (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 *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 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 Ian

Orme, Colorado State University. The cell stock was at a concentration of 5×10^6 CFU 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 s at 3,200 oscillations/min 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 (Tris-EDTA 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 10^9 CFU 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 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 min 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 *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 5% phenol. Briefly, each 1.5-ml trunk wash sample was spiked as described 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 noncommercial 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 FMD. Briefly, each 1.5-ml trunk wash sample was spiked as described above and then incubated for 30 min 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 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 μl of *M. avium* cells to yield a final spiking concentration of 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 completion of the appropriate trunk wash preparation steps, the samples were further purified and *M. bovis* DNA was extracted by 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 that already contained the remaining cell pellet and ~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 two pulses for 30 s at 3,200 oscillations/min. They were then incubated for 10 min 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 min (room temperature), and 400 μl of supernatant was transferred to one Zymo-Spin IV spin filter column. Samples were then treated according to the 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 IS*6110* 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 ~150 μl of supernatant that had been generated in the trunk wash preparation steps. To this, 15 2.5-mm zirconia beads (BioSpec) 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 two pulses for 30 s at 3,200 oscillations/min. After bead disruption, 40 μl of 20% sodium dodecyl sulfate and 14.85 μl 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 × *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 H_2O). Samples were vortexed for 30 s at maximum speed prior to centrifugation for 20 min at 11,000 × *g*. The 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 min was performed, and the clarified supernatant was transferred to a fresh low binding tube. An equal volume of cold 100% isopropanol was added, and the samples were thoroughly mixed via inversion or with a low-speed vortexer and then incubated at −70°C for 30 min (alternatively, samples may be stored overnight at −20°C prior to proceeding). Samples were centrifuged for 15 min at 11,000 × *g* (4°C) and washed twice. Each wash consisted of the addition of 800 μl of cold 70% ethanol, gentle resuspension of the pellet, centrifugation for 5 min 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 min or until DNA pellets were dry. The resulting pellets were rehydrated by the addition of 100 μl of Tris-EDTA buffer (Amresco, Inc., Solon, OH) and incubated for 20 min at 72°C. Extracted DNA was then ready for IS*6110* 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.

Noncommercial DNA extraction technique: column filtration. After completion of 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., Sugarland, TX). Each sample tube contained the remaining cell-soil pellet and ca. 150 μl of supernatant that had been generated in the trunk wash preparation steps. To this, 15 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], 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 μl of 20% sodium dodecyl sulfate and 14.85 μl of 20 mg of proteinase K/ml (final concentration, 0.675 mg/ml) 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 × *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 s at maximum speed prior to centrifugation for 10 min at 10,000 × *g* (room temperature). The resulting supernatants were transferred to one EconoSpin filter tube with attached lid (Epoch Biolabs) and centrifuged at 6,100 × *g* for 1 min and 15 s (room temperature). The filtrate was discarded, and

TABLE 1. Detection limits using the ZR and TSEP extraction techniques[a]

Treatment condition	No. of *M. bovis* cells[b]	
	ZR	TSEP
5% Phenol	5	10
Heat treatment (72°C)	1	10
M. avium spike	1	10

[a] Based on three replicate DNA extractions using the indicated treatment condition.
[b] Values represent the lowest concentration of *M. bovis* cells detected per 1.5-ml sample.

the filter was transferred to a fresh collection tube. A 500-μl portion of Wash ONE Buffer (5 M guanidine HCl and 20 mM Tris-HCl [pH 6.6] in final concentration of 38% ethanol) was added to the filter column, and the filter/collection tube was centrifuged at 6,100 × *g* for 1 min and 15 s. A final rinse was conducted using 750 μl of Complete Wash TWO buffer (20 mM NaCl, 2 mM Tris-HCl [pH 7.5], 80% ethanol), and the samples were again centrifuged. The filtrate was discarded, and the filter columns were centrifuged for an additional 1 min 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 min at room temperature, samples were centrifuged at 6,100 × *g* for 1 min and 15 s to elute the DNA. Extracted DNA was then ready for IS*6110* 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 IS*6110* insertion sequence of the *M. tuberculosis* complex. Extracted DNA was amplified by using conventional PCR targeting a 123-bp segment of the IS*6110* insertion sequence as previously described, with modifications (2, 3, 12). Briefly, each 25-μl reaction volume consisted of 1× AmpliTaq Gold Buffer II and 1.5 mM $MgCl_2$ (Applied Biosystems, Foster City, CA),0.2 mM concentrations of each nucleotide (Roche Applied Sciences, Indianapolis, IN), 0.4 μM concentrations of each primer (forward primer sequence, 5′-CCTGCGAGCGTAGGCGTCGG-3′; reverse primer sequence, 5′-CTCGTCCAGCGCCGCTTCGG-3′), 1.875 U of AmpliTaq Gold polymerase (Applied Biosystems), and 10 μl of DNA. Each reaction was overlaid with 30 μl of Chill Out wax (Bio-Rad, Hercules, CA) to prevent evaporation and placed into an MJ Research 60-place thermal cycler (Bio-Rad). The thermal cycling conditions consisted of an initial incubation at 94°C for 10 min to activate the polymerase, followed by 51 cycles of 94°C for 45 s and 72°C for 2 min and 15 s, with the final 72°C incubation being extended to 10 min. 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) 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 bp 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 nontuberculous mycobacteria and soil microbes. DNA was extracted from cultures of multiple species of nontuberculous mycobacteria and other soil microbes, including *M. abscessus* (ATCC 19977), *M. avium* (subsp. *hominissuis*, strain 2151), *M. avium* subsp. *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 by using fluorimetry, and 10 pg of DNA of each of the species was tested in the IS*6110*-targeted PCR protocol. As above, the laboratory technician who interpreted the electrophoretic results was blinded to the mycobacterial species present in each well.

Data analysis. The detection limit, or analytical sensitivity, for the IS*6110*-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 of 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.

All work was performed at the Animal Population Health Institute, College of Veterinary Medicine and Biomedical Sciences, Colorado State University, Fort Collins, CO.

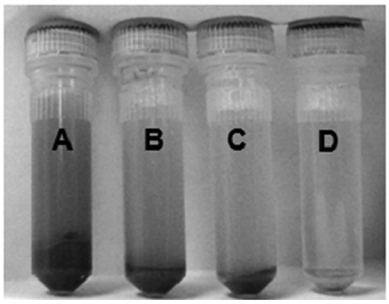

FIG. 1. Trunk wash samples (1.5 ml), obtained after the soil was allowed to settle in a 2.0-ml tube, with 300 mg of soil (A), 125 mg of soil (B), or 62.5 mg of soil (C), along with a normally acquired trunk wash sample with minimal soil contamination (D).

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

TABLE 2. Detection limits using the column filtration technique[a]

Soil sample (mg)[b]	No. of *M. bovis* cells[c]
300	50
125	20
62.5	5

[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] Values represent the lowest concentration of *M. bovis* cells detected.

TABLE 3. Reference guide and application summary for each DNA extraction technique evaluated

Test	Cost per sample (US$)	Process time (h)[a]	Soil tolerant[b]	Phenol tolerant	Heat tolerant	Analytical sensitivity[c]			
						Soil[d]	Phenol	Heat	*M. avium*[e]
ZR	4.20	2	500	Yes	Yes	1.25	5	1	1
TSEP	1.40	4	<62.5	Yes	Yes	NT	10	10	10
Column filtration	2.40	3.3	300	NT	NT	50	NT	NT	NT

[a] Time in hours based on 20 samples.
[b] Maximum soil (mg per 1.5-ml sample) allowable without inhibiting TB DNA detection.
[c] Values represent the lowest concentration of *M. bovis* cells (number of cells per 1.5-ml sample) detected.
[d] Based on maximum soil tolerance concentrations.
[e] When spiked with copious amounts of *M. avium*.
[f] NT, not tested.

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 IS*6110*-targeted PCR was evaluated after amplification of DNA extracted from cultures of multiple species of nontuberculous bacteria and other soil microbes (*M. abscessus*, *M. avium* subsp. *hominissuis*, *M. chelonae*, *M. fortuitum*, *M. intracellulare*, *M. marinum*, *M. avium* subsp. *paratuberculosis*, *M. phlei*, *M. simiae*, *M. smegmatis*, *M. szulgai*, *M. terrae*, *M. kansasii*, and *Nocardia asteroides).* 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 13 nontuberculous mycobacterial cultures, nor *Nocardia*, that were tested.

DISCUSSION

The results presented here 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 IS*6110* target sequence was amplified in samples spiked with *M. bovis* but not in samples spiked with *M. avium* or other nontuberculous mycobacteria. Using the extraction techniques outlined here, samples could be processed, and results were obtained in as few as 2 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 noncommercial 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. 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 IS*6110*-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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