

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

Meagan Kay², Lyndsey Linke^{1*}, Joni Triantis¹, R. Scott Larsen², M. D. Salman¹

Journal of Clinical Microbiology, 49 (2):618-623, 2011

¹Animal Population Health Institute, Colorado State University

²School of Veterinary Medicine, University of California, Davis, California

*Presenter: Lyndsey Linke



Tuberculosis in elephants

- Caused by *M. tuberculosis*, although *M. bovis* is also reported to infect these species
- Elephants lack clinical signs throughout most of the *M. tuberculosis* infection period
- 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



Current diagnostic methods

- Trunk wash culture serves as the “gold standard” for diagnosing TB in elephants
- Serological techniques useful for determining infection status
- Intradermal tuberculin test
- Experimental ELISA assay
- Nucleic acid extraction and amplification

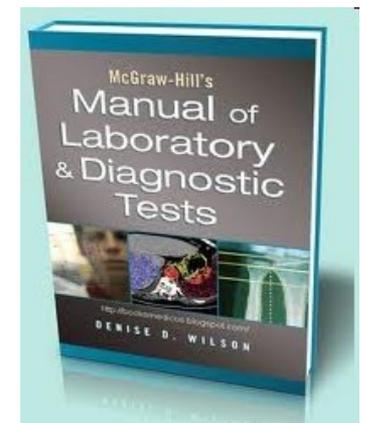


Trunk wash culture limitations:

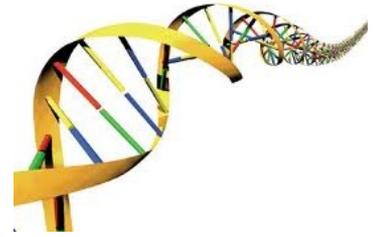
- Low sensitivity: requires > 100 organisms/ml for detection
- Can take up to 8 weeks for the bacteria to grow in culture
- Slow turn-around results in problematic travel restrictions and delays
- Susceptible to overgrowth from non-tuberculous mycobacteria or other organisms that may result in false-negative results

Characteristics of an ideal diagnostic test...

- Rapid
- Reasonable sensitivity/specificity
- Utilizes standard equipment and laboratory resources
- Ease of implementation and sample collection
- Does not generate hazardous waste
- Affordable



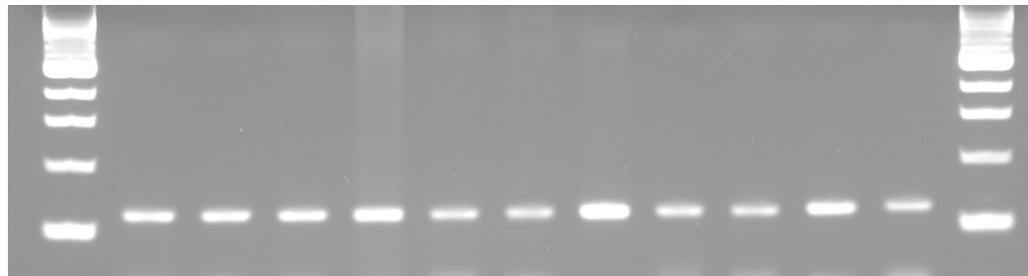
Molecular detection techniques



- Provide rapid and sensitive diagnostic technique for detecting TB in various clinical matrices
- Useful alternative/complement to culture for TB testing in elephants
- Molecular detection methods have not been fully evaluated or validated for elephant trunk washes

Combining diagnostic tools

Developing a DNA extraction method for trunk wash samples would allow *M. tuberculosis* detection directly in the exact material to which the “gold standard” is applied, providing a very useful diagnostic complement to the current “gold standard.”



Our criteria for developing molecular detection methods:

- Laboratory resources and equipment availability
- Potential factors present in samples that are inhibitory to PCR (soils, grains or grasses)
- Handling/treatment methods during shipping
- Presence of other mycobacterial species in trunk washes

Potential sample conditions and treatments considered during optimization:

- Clinical samples shipped from countries where FMD disease is endemic
 - Pre-importation treatment via heat inactivation at 72°C for 30 minutes (one of several recommended methods)
- Clinical samples shipped within US with *M. tuberculosis*-positive status
 - Treatment with 5% phenol prior to shipping
- Standard grazing/bathing habits resulting in contamination with soil and competing mycobacteria
 - Spike with large quantities of *M. avium* and known amounts of negative soil from elephant enclosure
- Clinical samples shipped on ice and stored frozen until processed
 - Subject spiked/treated samples to one freeze/thaw cycle at -70°C prior to DNA extraction procedures

Three DNA extraction techniques were designed and analyzed :

Commercial ZR soil microbe DNA kit (Zymo Research Corp., Irvine, CA) with slight modification

Non-commercial traditional salt and ethanol precipitation (TSEP) approach

Column filtration method with noncommercial buffers

ZR soil microbe DNA kit



- Technically simple, little manipulation required and short extraction period
- Does not generate hazardous waste
- No refrigeration required
- Greater cost due to commercial manufacturing
- Requires standard microcentrifuge, bead homogenization machine, and 100°C heating block
- Specialized reagents and filters to breakdown and remove polyphenols and humic acids present in soil



Traditional salt ethanol precipitation (TSEP)

- More cost efficient due to homemade reagents
- Longer extraction period
- Technically more challenging with more manipulation required
- Does not generate hazardous waste
- Requires refrigerated microcentrifuge, bead homogenization machine, 65°C and 100°C heating blocks, 4°C refrigeration, -20°/-70°C freezer

Column filtration method



- More cost efficient due to homemade reagents
- Commercial filter column (EconoSpin filter, Epoch Biolabs, Inc.)
- Uses no hazardous reagents
- Short extraction period with moderate manipulation
- No refrigeration required
- Homemade reagents and column filter circumvent inhibitory effects of soil
- Requires standard microcentrifuge, bead homogenization machine, and 65°C and 100°C heating blocks

Materials and Methods



Preparation of trunk wash samples for method optimizations

- Trunk washes acquired from 2 adult captive female Asian elephants (alternating weeks over 6-month period)
- Culture consistently negative for *M. tuberculosis* complex bacteria (NVSL)
- Samples pooled to create homogenous matrix for spiking trials and aliquoted into 1.5ml volumes for spiking and extraction

Spikes and negative extraction controls

Cell stocks of killed *M. bovis* (strain 846146, kindly provided by Ian Orme, Colorado State University) were used for spiking

- *M. bovis* cells diluted to reflect theoretical [] of 100, 50, 20, 10, 5, 1 cell per 1.5ml trunk wash sample
- Negative extraction controls consisted of samples spiked with dilution buffer (0 cells)
- Samples were subjected to appropriate treatment conditions
- Samples were frozen at -70°C, thawed, and centrifuged
- Supernatant was removed to ensure exact volume of 150 µl remained for extraction

Phenol Treatment



Mimic treatment required for U.S. domestic shipment of known *M. tuberculosis*-positive samples

Phenol treatment tested with commercial ZR technique and noncommercial TSEP technique

1. Samples were spiked as previously described
2. Each 1.5-ml trunk wash sample was treated with molecular-grade phenol to 5% final concentration
3. Samples were frozen at -70°C , thawed, and centrifuged
4. 150 μl remained for subsequent DNA extraction

Heat Treatment

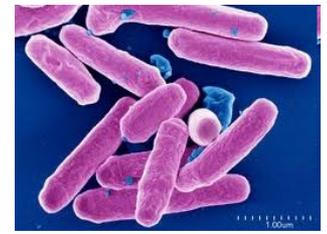


Mimic recommended treatment for samples originating from countries endemic with FMD

Heat treatment tested with commercial ZR technique and noncommercial TSEP technique

1. Samples were spiked as previously described
2. Each 1.5-ml sample was incubated for 30 minutes at 72°C
3. Samples were frozen at -70°C, thawed, and centrifuged
4. 150 µl remained for subsequent DNA extraction

Contamination with *M. avium*



Mimic presence of competing mycobacteria

Tested with commercial ZR and noncommercial TSEP technique

1. Samples were spiked with *M. bovis* as previously described
2. Each 1.5-ml sample was additionally spiked with *M. avium* cells (10^8 cells per 1.5-ml sample)
3. Samples treated with 5% phenol (mimic harshest treatment)
4. Samples were frozen at -70°C , thawed, and centrifuged
5. 150 μl remained for subsequent DNA extraction

Contamination with soil



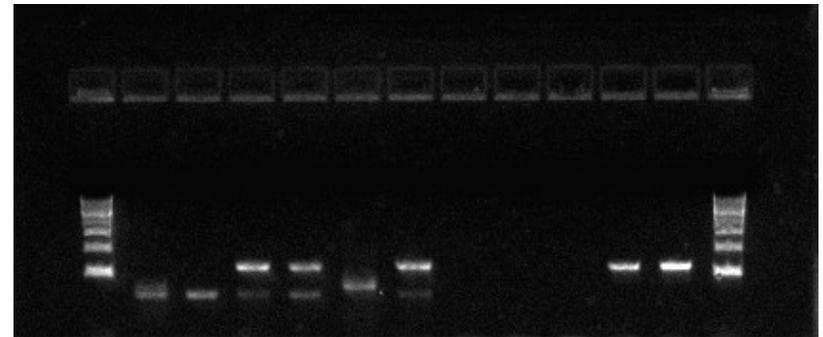
Mimic presence of PCR inhibitory organic soil material

Soil contamination tested with all three DNA extraction techniques

1. Each 1.5-ml trunk wash sample was spiked with 300, 125, or 62.5 mg of soil
2. Samples were spiked with *M. bovis* as previously described
3. Samples were frozen at -70°C , thawed, and centrifuged
4. 150 μl remained for subsequent DNA extraction

Conventional PCR amplification

- PCR targeting 123-bp segment of IS6110 insertion sequence of *M. tuberculosis* complex
- Agarose gel electrophoresis using the FlashGel DNA System (Lonza Group, Ltd., Basel Switzerland)
- Samples producing expected band size of 123 bp were considered positive; bands any other size considered negative



IS6110 PCR Specificity Verification

Specificity Panel	IS6110
<i>M. abscessus</i> (ATCC 19977)	negative
<i>M. avium</i> (subsp. <i>hominissuis</i> , strain 2151)	negative
<i>M. avium</i> subsp. <i>paratuberculosis</i> (ATCC 19698)	negative
<i>M. chelonae</i> (ATCC 35752)	negative
<i>M. fortuitum</i> (ATCC 19542)	negative
<i>M. intracellulare</i> (ATCC 13950)	negative
<i>M. kansasii</i> (ATCC 12478)	negative
<i>M. marinum</i> (ATCC 927)	negative
<i>M. phlei</i> (ATCC 11758)	negative
<i>M. simiae</i> (ATCC 25273)	negative
<i>M. smegmatis</i> (ATCC 23011)	negative
<i>M. szulgai</i> (ATCC 35799)	negative
<i>M. terrae</i> (ATCC 15755)	negative
<i>Nocardia asteroides</i> (ATCC 3308)	negative
<i>M. bovis</i> (strain 846146)	positive

DNA extracted from cultures of multiple species of non-tuberculous mycobacteria and other soil microbes

DNA was quantified and tested with the IS6110-targeted PCR protocol

Data analysis

- Analytical sensitivity (detection limit) for IS6110-targeted PCR, for each extraction technique was reported as lowest [] of *M. bovis* cells detectable in 3/3 trials
- Analytical specificity was ability to only produce 123-bp product when spiked with *M. bovis*
- Both estimates reported as absolute figures rather than proportions (no statistical inferences were performed)

Results



Detection limits: ZR and TSEP technique

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

Treatment condition	No. of <i>M. bovis</i> cells ^b	
	ZR	TSEP
5% Phenol	5	10
Heat treatment (72°C)	1	10
<i>M. avium</i> 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.

Lowest detection level in 3/3 replicates spiked with *M. bovis* that produced 123 bp product after DNA extraction, under each treatment condition

Detection limits: soil contamination

- Noncommercial column filtration method analyzed in presence of soil
- ZR method routinely demonstrated 1.25 *M. bovis* cells per 500 mg of soil (data not shown)
- TSEP method greatly compromised with ≥ 62.5 mg of soil; not further evaluated

TABLE 2. Detection limits using the column filtration technique^a

Soil sample (mg) ^b	No. of <i>M. bovis</i> 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.

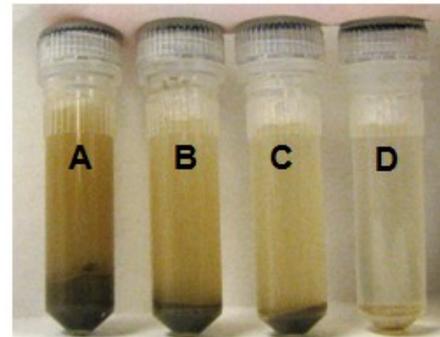


FIG. 1. Trunk wash samples (1.5 ml), obtained after the soil was allowed to settle in a 2.0-ml tube, with 300 mg of soil (A), 125 mg of soil (B), or 62.5 mg of soil (C), along with a normally acquired trunk wash sample with minimal soil contamination (D).

DNA extraction technique summary

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	<i>M. avium</i> ^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.

- Robust utility of ZR and TSEP techniques – high tolerance to various treatments
- *M. avium* does not affect detection limits of ZR or TSEP
- Noncommercial column filtration technique provides viable alternative to commercial ZR method for processing samples heavily saturated with soil
- Samples could be processed, and results obtained in as few as 2 days after clinical submission, while providing a cost-effective diagnostic assay

Conclusions

Initial results of our study suggest that these 3 extraction techniques are:

1. Highly sensitive and specific for *M. tuberculosis* complex
2. Easily and rapidly performed
3. Adaptable to laboratories with limited resources
4. Robust performance under variety of conditions



These techniques provide promise for detecting *M. tuberculosis* complex in Asian elephants

Future

- Determine if these techniques can be used for future evaluation of TB infection status in elephants
- Must validate with clinical samples from infected animals to compare to culture
- DNA extraction and PCR analysis could be part of a battery of tests, including culture and serology



Acknowledgements

Funding:

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

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



Questions?

