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Rapid screening for *Mycobacterium tuberculosis* complex in clinical elephant trunk wash samples

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ABSTRACT

Mycobacterium tuberculosis can infect and be transmitted between elephants and humans. In elephants, the 'gold standard' reference test for detection of tuberculosis is culture, which takes a minimum of eight weeks for results and has limited sensitivity. A screening test that is rapid, easily implemented, and accurate is needed to aid in diagnosis of tuberculosis in elephants. Ninety-nine clinical trunk wash samples obtained from 33 elephants were utilized to validate three molecular extraction techniques followed by a polymerase chain reaction for detection of *M. tuberculosis*. Diagnostic sensitivity and specificity were estimated compared to culture. Kappa coefficients were determined between molecular results and various culture categories and serological test results. An internal amplification control was developed and assessed to monitor for PCR inhibition. One molecular test (the Column method) outperformed the other two, with diagnostic sensitivity and kappa agreement estimates of 100% (CI 57–100) and 0.46 (CI 0.2–0.74), respectively, compared to culture alone. The percentage of molecular-positive/culture-negative samples was 8.4% overall. The molecular extraction technique followed by PCR provides a much-needed rapid screening tool for detection of tuberculosis in elephants. Immediate procedures can be implemented to further assess PCR-positive animals and provide personnel biosecurity. While a positive result is not a definitive test for elephant tuberculosis, the molecular test results can be used to support current diagnostic procedures applied by veterinarians for treatment decisions to prevent the spread of tuberculosis in elephants.

1. Introduction

Bacterial organisms in the *Mycobacterium tuberculosis* complex (MTBC), particularly *M. tuberculosis* (MTB) and *M. bovis*, cause tuberculosis (TB). Elephants are susceptible to mycobacterial infections, most commonly with *M. tuberculosis*, although *M. bovis* and rare cases of infection with atypical mycobacteria such as *M. szulgai*, *M. avium*, and *M. elephantis* have been reported (Lacasse et al., 2007; Yong et al., 2011). Tuberculosis has been detected in captive Asian elephants (*Elephas maximus*) in North America since before 1996 (Mikota, 2008). In the United States, the disease had a median point prevalence of 5.1% in Asian elephants between 1997 and 2011 (Feldman et al., 2013).

While some infected elephants develop clinical signs of TB, most are asymptomatic, but importantly, are potentially still able to shed the bacteria (Mikota et al., 2000). It is therefore assumed that elephants and humans can contract the infection from both clinically and sub-clinically affected animals that are shedding MTB through aerosolized trunk secretions containing mycobacteria (Mikota et al., 2001). Undiagnosed tuberculosis in elephants that are shedding MTB could put other animals and workers at risk of infection (Michalak et al., 1998; Murphree et al., 2011). Confirmed zoonotic transmission of MTB between elephants and humans has been recorded in elephant workers (Michalak et al., 1998; Zlot et al., 2016). Thus, rapid diagnosis of mycobacterial shedding is an essential step in effective management of tuberculosis in elephant populations and control of this novel occupational risk.

Screening tests that are easily implemented, cost-effective, and accurate are required for early diagnosis of TB (Mikota et al., 2000). Although trunk wash culture currently serves as the 'gold standard' for TB diagnosis in elephants with high specificity and positive predictive values, (Mikota et al., 2001), culture is not without serious limitations. Overgrowth of non-tuberculous organisms (Mikota et al., 2000; Tsai and Olson, 1992) contributes to the low sensitivity of culture, and thus individual negative culture results are considered non-definitive, as they cannot rule out a TB infection (USDA, 2010). While TB is chronic and transmission occurs slowly, elephants can potentially shed bacteria over a long period (Mikota et al., 2015) and there is a risk for TB to spread to other animals during the extended time frame (8 weeks) necessary to obtain culture results (Fowler, 2006). Travel restrictions imposed due to delayed laboratory results during culture processing can adversely affect zoological institutions or circuses, which rely heavily

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on interstate travel. Other tests for tuberculosis, such as the skin test, are unreliable in elephants, or, in the case of serological tests, are considered to be secondary tests with poor sensitivity and specificity in humans (WHO, 2011; Achkar et al., 2011; Steingart et al., 2012) and with mixed results in elephants (Elephant Tuberculosis Research Workshop, 2005; Larsen et al., 2000; Greenwald et al., 2009; Lyashchenko et al., 2006). Serology can be informative for determining whether exposure has occurred, but does not identify the presence of *M. tuberculosis*-complex organisms in the animal. Furthermore, antibody titers may also remain detectable even after treatment (Isaza and Ketz, 1999; Larsen et al., 2000; Lyashchenko et al., 2006).

Mycobacterium tuberculosis-complex bacteria have been found in naturally contaminated soil (Hruska and Kaevska, 2012; Velayati et al., 2015) and can be detected rapidly and sensitively by extraction of nucleic acids and subsequent polymerase chain reaction (PCR) analysis from spiked soil (Ghodbane et al., 2014), feces (Balamurugan et al., 2006; Khéchine et al., 2009; Wolf et al., 2015), tissues (Kay et al., 2011; Miller et al., 2002; Thacker et al., 2011), and nasal swabs (Crawshaw et al., 2014; Palmer et al., 1999; Vitale et al., 1998). Use of these molecular methods could provide a valuable alternative or complement to trunk wash culture for MTBC screening in elephants.

The aim of this study was to estimate the diagnostic sensitivity, specificity and kappa agreements of three molecular extraction techniques followed by PCR for rapid detection of MTBC in elephant trunk washes, compared with the trunk wash culture. A potential method for monitoring the presence of inhibitors in trunk wash samples was also investigated. Results of the molecular methods were also compared with comprehensive culture categories and other existing conventional tests for the diagnosis of or screening for MTBC in elephants.

2. Materials and methods

2.1. Samples, culture and serology

Samples were obtained from captive Asian elephants in the US during routine annual TB screening events and as such were exempt from Institutional Animal Care and Use requirements. Institutional Biosafety Committee approval was obtained for storage and assay of the samples. Valid trunk wash specimens were obtained from all animals using USDA standard collection techniques (Isaza and Ketz, 1999; USDA, 2010). All elephants were compliant and no adverse events were noted. Ninety-nine clinical trunk wash samples were obtained from 33 elephants from three collection days. The samples were obtained from one herd that was likely to contain culture-positive elephants, according to the history of some animals in the herd having been previously culture-positive (and treated). At the time of sample collection for this study the culture status of individual animals was unknown. The volume of each clinical sample was split at the time of collection for culture and molecular testing. The samples were aliquoted and frozen at −80 °C before being shipped frozen to the laboratory.

Culture was conducted according to the USDA guidelines (USDA, 2010) by trained and experienced personnel at the Mycobacteriology Laboratory at National Jewish Medical and Research Center in Denver, CO. A sample was considered culture-positive upon isolation of *M. tuberculosis*. Veterinarians at the elephant premises used two commercial lateral-flow devices (DPP and STAT-PAK, ChemBio Diagnostics, Medford, NY) to perform serological testing on collected serum samples. Serum samples were in most cases obtained within 1–2 weeks of the trunk wash samples. Any serum sample positive by the lateral flow device(s) was subsequently tested using a multiple antigen print immunoassay (MAPIA, manufactured and conducted by ChemBio Diagnostics, Medford NY).

Upon receipt for molecular testing, trunk wash samples were aliquoted and stored frozen at −80 °C. Subsequent DNA extractions and PCR assays were performed by trained laboratory personnel at the Animal Population Health Institute at Colorado State University who were blinded to all culture, serological, and TB history status.

It is noted that shedding of TB in elephants is most often intermittent in nature (Vogelsnest et al., 2015; Feldman et al., 2013), and thus culture results may vary not only from day-to-day but from week-to-week. Therefore, in addition to comparison to standard culture (culture and DNA obtained from the same aliquot of trunk wash material), 3 broad culture categories were utilized as an indirect means to evaluate the risk for shedding MTBC organisms. A wider picture of the relation of the molecular test results to current (individual 3 tests within 1 week) as well as the recent and past TB culture history of the elephants was thus developed. Accordingly, the term 'Culture Cluster' is utilized to describe: if any of the 3 sequential samples obtained during the current study (3 within 1 week) were culture-positive, the status of that animal and all 3 sequential samples from that animal were considered as culture-positive for comparison to molecular results. Routine TB culture testing results (from periods other than the collection for this study) were utilized to categorize the recent and overall culture history of an animal. If an animal had been found to be culture-positive during routine testing within 1 year after collection for the current study, it was considered 'TB-Recent'-positive for comparison to the molecular results. Similarly, if an animal had been found to be culture-positive during routine testing at any time during its recorded history, it was considered 'TB-History'-positive (regardless of treatment) for comparison to the molecular results.

2.2. DNA extractions and PCRs

Trunk wash samples, previously tested as culture- and PCR-negative from another herd (Kay et al., 2011), were pooled and aliquoted into appropriate volumes for use as extraction controls. A cell stock of rinsed, heat-killed *M. bovis* cells was used for spiking the pooled negative trunk wash material to serve as positive extraction controls as follows. On the day of each extraction, 50 μL of *M. bovis* cells (100 cells) were spiked into a 5 mL culture-negative trunk wash pool aliquot. Negative extraction controls were prepared similarly, utilizing 50 μL of dilution buffer only.

Five milliliters of each clinical sample was thawed at room temperature and centrifuged (20 min, 4800 ×*g*, 4 °C) along with the prepared controls, after which 4.5 mL of supernatant was discarded. The pellets were thoroughly suspended in the remaining 0.5 mL of supernatant and transferred to a bead beater tube (Sarstedt, Nümbrecht, Germany). The initial tube was then rinsed with 0.5 mL phosphate buffered saline (PBS) to obtain any residual pelleted material, which was added to the bead beater tube, before centrifugation for 20 min at 11,000 ×*g* (4 °C). An estimate of the soil load in each sample was made by height and width measurement of the soil pellet, and by comparison to a visual standard curve with weighed soil added. Supernatant was carefully removed, leaving 150 μL of liquid so as to completely avoid inadvertent removal of any pelleted material. Extractions by the three techniques, ZR, (Zymo Soil Microbe DNA kit, Zymo Research, Irvine CA), Column (an in-house technique utilizing a commercial silica membrane column (Epoch Life Science, Sugar Land TX)), and TSEP (in-house technique utilizing a traditional salt and ethanol precipitation), were conducted as previously described (Kay et al., 2011).

All DNA elutions were performed into low-binding tubes (Life Science Products, Denver CO). The DNA from each of the three extraction methods was tested by IS6110 PCR as previously described (Kay et al., 2011), with minor modifications. Briefly, DNA was tested undiluted, 1:2, and 1:5 by IS6110 PCR after extraction by each of the three methods. Additionally, 0.2 μg of bovine serum albumin (BSA; Amresco, Solon OH) was added per 25 μL reaction. PCR reactions with 10 femtograms (fg) of purified *M. bovis* DNA and molecular grade water were included as positive and negative PCR controls, respectively. The term 'molecular method' herein refers to the particular extraction technique followed by IS6110 PCR.