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Title: Serodiagnosis of elephant tuberculosis: A useful tool for early identification of infected elephants at the captive-wild interface

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Abstract Tuberculosis (TB) is an emerging disease in elephants primarily caused by *Mycobacterium tuberculosis* (*M. tb)* and in some occassion by *M. bovis*. We performed culture and three serological tests –the Elephant TB STAT-PAK,® DPP VetTB® Assay, and MAPIA (multi-antigen print immunoassay)–prospectively on samples from eight elephants in Nepal that died of suspected or confirmed tuberculosis (TB) between 2007 and 2013. Among them, all elephants were reactive to DPP VetTB® Assay, five to Elephant TB STAT-PAK,® and two were reactive to MAPIA. Similarly, six elephants were positive on culture on samples collected antemortem or post-mortem. We observed antibody responses months to years before culture confirmation of TB which shows that serological tests can be highly useful for the early diagnosis of TB in elephants. Validated point-of-care serological tests are easily performed in the field and hold promise for improved TB surveillance in other non-domestic species.

Key words Asian elephants, tuberculosis, DPP VetTB Assay, ElephantTB STAT-PAK, serological assay.

Introduction

Tuberculosis (TB) is an emerging disease in elephants caused primarily by *Mycobacterium tuberculosis* (*M. tb).* Numerous cases have occurred among captive elephants, but it is only since 2013 that *M. tb* has been found in wild elephants (Obanda et al. 2013; Perera et al. 2014; Zachariah et al. 2017; Chandranaik et al. 2017). The occurrence of this human pathogen in the wild is cause for concern and further research.

In captive elephants, TB is diagnosed by isolating *M. tb* from respiratory samples collected using a trunk wash procedure (Lyashchenko et al. 2006). This method has been widely used in the U.S. and Europe. In elephant range countries, *M. tb* has been cultured from captive elephants in India (Venugopal and Abraham 2015), Nepal (Paudel et al. 2014), Thailand (Angkawanish et al. 2010), and from an elephant in Australia that was imported from Thailand (Stephens et al. 2013). Although positive culture provides a definitive diagnosis, the method has significant limitations (Lyashchenko et al. 2006; Mikota et al. 2015). In many cases, repeated ante-mortem culture fails to identify infected elephants that are then diagnosed postmortem. Supplementary Table 1 lists publications that rreport the poor recovery of *M. tb* from multiple trunk wash samples from known TB-infected elephants.

Specific serological point-of-care tests offer a practical TB screening method in elephants and other wildlife species. The ElephantTB STAT-PAK® (STAT-PAK) and Dual Path Platform (DPP) VetTB® assays (Chembio Diagnostic Systems, Inc., Medford NY, USA), licensed by the United States Department of Agriculture, have been used for TB screening in several Asian elephant range countries (Abraham et al. 2008; Mar et al. 2012; Ong et al. 2013; Lassausaie et al. 2015). The more accurate DPP replaced the STAT-PAK in 2012.

TB was first diagnosed in elephants in Nepal in 2002 and between 2002 and 2015, 13 elephants died of suspected or confirmed TB. The Nepal Elephant TB Control and Management Action Plan was initiated in 2011 to mitigate the transmission of TB between free-ranging wildlife such as rhinoceros and wild elephants and captive elephants used for tourism, patrolling of protected areas, and research (Mikota et al. 2016). This Plan was approved by the Department of National Parks and Wildlife Conservation, the Ministry of Forestry and provides guidelines for routine testing, treatment, and reintegration of elephants into active service following treatment when possible.

We present the clinical, serological, and culture data from eight cases and show that specific serological tests are a useful tool to manage TB in captive elephants and decrease TB risk to wild populations.

Materials and Methods

Study subjects and sample collection

Four male and four female captive elephants, aged 31–70 years were included in the study (Supplementary Table 2). Seven dead elephants were owned by the government and one elephant was privately owned. Health care and clinical examination was provided by Elephant TB program veterinarians, supervised by the government's Senior Wildlife Veterinarian. All animals died from suspected TB based on necropsy lesions and none was euthanized.

Pre-mortem blood was collected from the auricular vein of seven elephants. Twenty-one trunk wash samples for culture were collected at various times ante-mortem from six elephants according to published guidelines (USAHA 2010) or using a modified procedure (Abraham and

Davis 2008). In one case (E8), an excreted respiratory discharge sample was submitted for culture.

Serology testing

The multi-antigen print immuno assay (MAPIA) was originally developed as a tool to identify seroreactive antigens in human TB and has been adapted to identify the immunodominant proteins of *M. tb* or *M. bovis* recognized in elephants and other species (Lyashchenko et al. 2006). The selected antigens were used for the development of the serological tests described below. In this study, the MAPIA was performed at the Chembio laboratory on serum samples collected in 2006 from six elephants (E1, E2, E3, E6, E7 and E8) as previously described (Lyashchenko et al. 2006).

The STAT-PAK is a lateral flow screening test that incorporates a cocktail of several *M. tb* and/or *M. bovis* antigens impregnated on nitrocellulose membrane housed in a disposable plastic cassette. Sample and buffer solution migrate to a test pad by capillary action. If antibodies are present, they bind to the antigen and the colored latex bead-based signal detection system results in a visible blue line in the test window (Lyashchenko et al. 2006).

The DPPVetTB Assay is an immune-chromatographic screening test that detects antibodies to ESAT-6/CFP 10 and MPB83 antigens. The DPP technology uses two nitrocellulose strips allowing independent delivery of the test sample and antibody-detecting agent of colloidal gold particles (Greenwald et al. 2009). The ElephantTB STAT-PAK® and DPP VetTB® assays (Chembio Diagnostic Systems, Inc., Medford, NY) were performed according to the manufacturer's instructions using blood collected ante-mortem or banked serum. In two cases (E5 and E7) the DPP was performed using frozen-thawed post-mortem lung fluid.

Postmortem examination

Post-mortem examinations were performed on seven elephants. Elephant E4 was returned to her owner in India where she died after several months; a postmortem examination was not performed. All staff used personal protective equipment including N-95 masks during necropsy. The necropsy was performed using procedures recommended by Montali (2006). Representative lung lesions were collected in sterile tubes for culture. Tissue samples from elephant E1 were submitted to the Central Veterinary Laboratory in Kathmandu, Nepal for histopathology.

Culture of trunk discharges and lung tissues

Cultures of lung tissue samples were conducted following the guidelines of European Society for Mycobacteriology (Groothius and Yates 1991) and Lowenstein- Jensen (L-J) media was used to culture the trunk wash samples and lung lesions. Decontamination procedure was used according to (USAHA, 2010).

Results

The relationship between serology and culture results of eight elephants to time of death is illustrated in Figure 1. Clinical, diagnostic, postmortem, and culture data of eight TB-suspect elephants in Nepal is presented in Supplementary Table 2. Five of eight elephants showed clinical signs of TB including coughing, trunk discharge, or weight loss.

Serological testing

Two of six elephants tested by MAPIA in 2006 were seropositive (E2 and E3). E3 was seropositive more than three years prior to diagnosis by culture. Elephants E6, E7, and E8 were non-reactive on MAPIA in 2006, but showed positive reactivity on the DPP test and had evidence of TB at postmortem examination 6-7 years later, suggesting that they were infected and seroconverted after 2006.

Between 2006 and 2012, seven elephants were tested using the STAT-PAK. Elephants E1, E2, E3 and E4 were seropositive, elephant E7 had equivocal results, and elephants E6 and E8 were seronegative.

All eight elephants reacted on the DPP VetTB assay at some point between 2006 and 2013, including two elephants that were tested using postmortem lung fluid. This is an off-label use of the test but shows that TB antibodies are present in other body fluids.

Postmortem

All seven elephants that underwent postmortem examination had gross lung lesions compatible with TB. Histopathology performed on tissues from E1 showed acid-fast bacilli in the lung, scattered granulomatous foci, and a chronic inflammatory response.

Culture

Twenty-one trunk wash samples collected antemortem from six elephants were negative for *M. tb*. Five lung tissue samples and one pre-mortem trunk wash sample from six elephants (E3-E8) were culture positive for *M. tb*. Ante-mortem trunk respiratory discharge from E4 was positive on culture shortly before her death. Elephant E1 was negative on ante- and postmortem cultures despite gross postmortem findings compatible with TB. Elephant E2 also had distinct TB-like lung gross lesions at postmortem examination; however, samples were not submitted for culture.

Discussion

In our study, TB was confirmed at the time of death by culture in six cases. Culture was negative in the case of E1, however, samples may have been compromised during shipment because of high ambient temperatures. Samples for culture were transported to the TB laboratory within 24 h of collection. Culture was also negative for TB in all cases where it was performed at times significantly preceding death and consequently was not a predictor of disease.

By contrast, Elephants E3 and E4 were reactive on the STAT-PAK 44 and 28 months respectively before diagnosis by culture, supporting previous findings that elephants can be seropositive months to years in advance of TB diagnosis by culture (Lyashchenko et al. 2012). We did not detect seroconversion in E6 and E7 until shortly before they died of TB.

We observed a strong correlation between serology and post-mortem confirmation by culture and poor correlation between antemortem trunk wash culture and post-mortem diagnosis. In our study, 21 trunk wash samples collected at various times from six elephants were negative for mycobacteria; only the expectorated discharge from E4 was positive shortly before her death. All the elephants that were culture positive postmortem were reactive on one or more serological tests but antemortem trunk wash samples were never culture positive (Figure 1).

Other studies have reported similar findings (Supplementary Table 1). In Thailand, *M. tb* was isolated from only two of 60 trunk wash samples from three elephants that were culture positive post-mortem (Angkawanish et al. 2010). In Sweden, only 7 of 189 trunk wash sample were positive from 5 elephants confirmed TB-infected at postmortem (Moller et al.2005). Although trunk wash culture is considered the “gold standard” for the diagnosis of TB in elephants, these examples clearly show the deficiencies of culture for surveillance and intervention.

There are 109 to 142 resident wild elephants and several elephant herds that migrate between Nepal and India (Pradhan et al. 2011). Captive and wild elephants interact during natural breeding and intermingle with other wildlife during grazing in protected areas. Captive elephants have close contact with wild rhinos during tourist safaris, patrolling, translocations, and during the annual rhino count which is conducted on elephant-back. This provides ample opportunities for TB to be transmitted to endangered wildlife and the potential for the development of a wild reservoir of *M. tb*.

While *M. bovis* is a known threat to a variety of species in the wild such as Eurasian badgers (*Meles meles*) (Drewe et al. 2010), and cape buffalo (*Syncerus caffer*) where in Kruger National Park TB has spilled over to other mammalian species, including lions (Miller et al. 2012), it is only since 2013 that *M. tb* has been found in any wild species. A wild African elephant that had been under human care was the first reported case although it is not clear whether the mycobacterial species was confirmed (Obanda et al. 2013). Since then, four cases with no history of captivity have been reported in wild Asian elephants in India (Zachariah et al. 2017; Chandranaik et al. 2017) and one additional case in Sri Lanka (Perera et al. 2014).

When successful, culture and isolation of *M. tb* is valuable to confirm infection, identify the mycobacterial species and strain, and test it for drug susceptibility (Mikota 2008). But in developing countries culture method is not practical, nor reliable, as a primary technique for TB screening.

The serological tests have been shown to be early and accurate correlatives of active TB in elephants (Lyashchenko et al. 2006, 2012). Prompt diagnosis of TB at the captive-wild interface facilitates initiation of appropriate management strategies to prevent transmission to herd mates, wild elephants, rhinos, and other susceptible species, including humans. These tests have been instrumental in the success of the Nepal Elephant Tuberculosis Control and

Management Action Plan. Through on-going surveillance and treatment, no deaths related to TB were reported between 2013 and 2018.

The value of these serological tests is not limited to elephants although their use in most of other wildlife species is off-label. The DPP VetTB Assay has been used in rhinos (Duncan et al. 2009; Miller et al. 2015), lions (Miller et al. 2012), warthogs (Miller et al. 2016), badgers (Drewe et al. 2010), and wood bison (Himsworth et al. 2010). In the United States, the DPP VetTB Assay is approved by the USDA Bovine TB Eradication Program for testing several species of captive cervids (Lyashchenko et al. 2013, 2018). This study shows serology as promising "non-culture" based TB diagnosis method in elephants, however, more samples size are required to determine a statistical correlation between serology and culture in elephants.

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Fig 1 Relationship of serology and culture results to time of death

Footnote: serology shown includes results of one or more tests during the time period indicated. The MAPIA was only run in 2006.

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Table 1. Reports Demonstrating the Poor Recovery of TB from Multiple Trunk Wash Samples From Known TB-Infected Elephants

Country	Number of elephants with confirmed TB	Number of trunk washes performed	Number of TB-positive cultures	Reference
Sweden	5	189	7	Moller et al. 2005
Thailand	3	60	2	Angkawanish at al. 2010
Australia	1	371 (includes samples collected from 6 elephants total)	1	Vogelnest et al. 2015
Switzerland	3	> 200 (includes samples collected from 10 elephants total)	0 (3 elephants were euthanized and TB confirmed postmortem)	Steinmetz and Rutten 2016

Table 2. Clinical serological and culture data of eight elephants in Nepal

Elephant	Gender	Age (Years)	Clinical Signs	MAPIA Results	STAT-PAK Results	DPP Results	AM culture results	PM culture results	Date of Death	Comments
E1	M	47	weight loss; reflux	NR 2006	R 2006	R 2006 (banked serum)	N 2006 (n=3)	Negative	Aug 2007	Suspect culture samples damaged during transport
E2	M	32	weight loss; coughing	R 2006	R 2006	R 2006 (banked serum)	N 2006 (n=3)	NA	Sept 2007	Samples for culture not submitted
E3	F	65	weight loss	R 2006	R 2006, 2008	R 2009	N 2006 (n=2) N 2008 (n=1)	*M. tb*	Aug 2009	Too old to treat
E4	F	56	coughing, weakness, trunk discharge	NA	R 2008, 2010	R 2010	P 2010 (n=1) *M.tb*	NA	2010; month unknown	*M.tb* isolated from trunk discharge; no PM or PM culture performed
E5	F	60	none	NA	NA	R 2009 (lung fluid)	NA	*M. tb*	Sept 2009	AM blood not available
E6	M	31	none	NR 2006	NR 2006, 2008, 2010, 2011	R 2012	N 2006 (n=3) N 2010 (n=1)	*M. tb*	Sept 2012	Extensive TB lesions; late to sero-convert compared to other cases
E7	F	65 or 70	loss of muscle mass noted at necropsy	NR 2006	NR 2006, 2008, 2010; equivocal 2011	NR 2011; R 2013 (lung fluid)	N 2006 (n=3) *M. terrae* isolated from one sample	*M. tb*	Feb 2013	Extensive TB lesions; late to sero-convert compared to other cases
E8	M	32	weight loss	NR 2006	NR 2006, 2007, 2008	R 2013	N 2006 (n=3) N 2013 (n=1)	*M. tb*	March 2013	Five year serology testing gap

R: reactive ; NR: non-reactive ; N: negative ; P: positive ; NA: not applicable ; AM: Ante-mortem ; PM : Post-mortem

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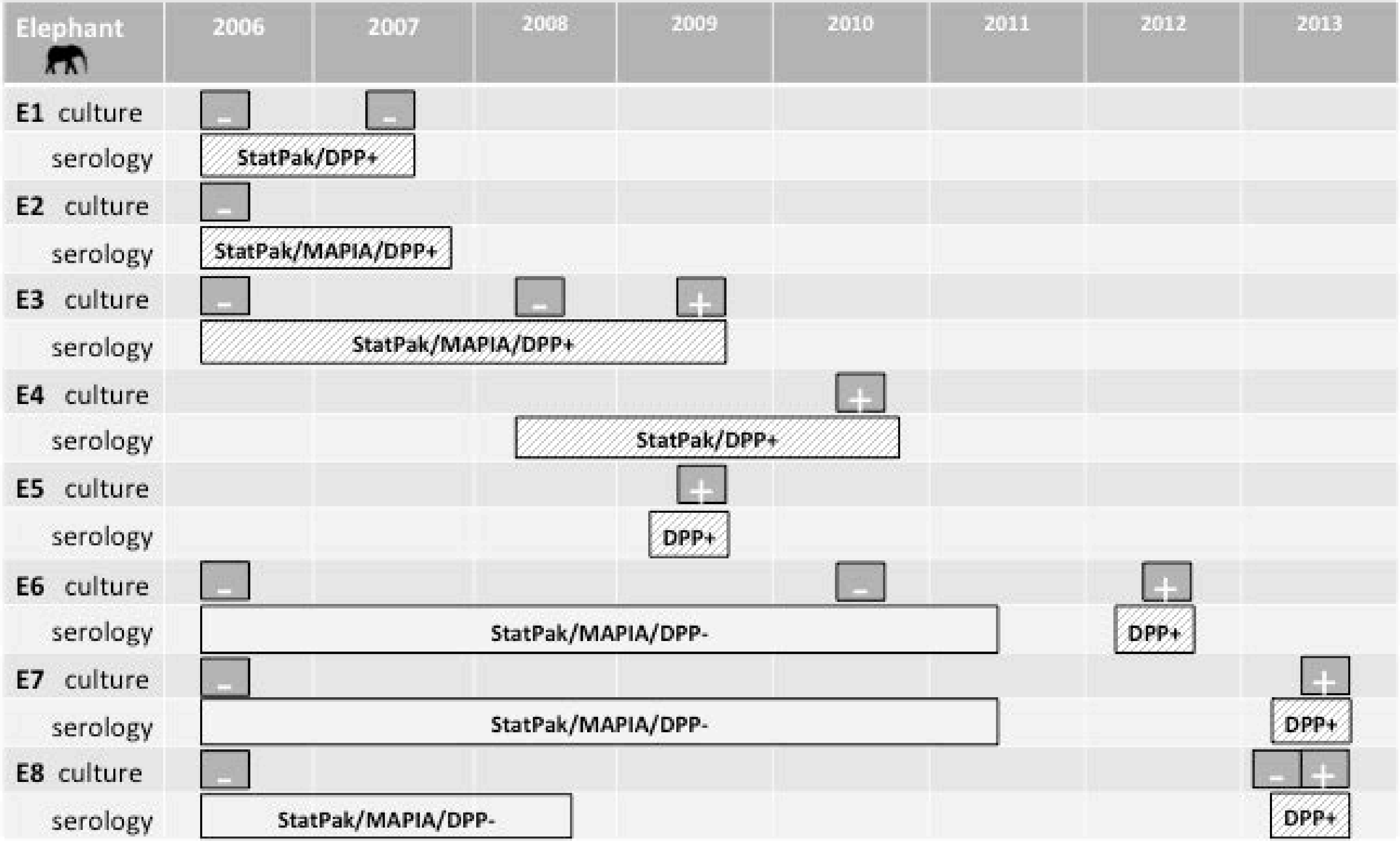
Elephant
2006
2007
2008
2009
2010
2011
2012
2013
E1 culture
serology
StatPak/DPP+
E2 culture
serology
StatPak/MAPIA/DPP+
E3 culture
serology
StatPak/MAPIA/DPP+
E4 culture
serology
StatPak/DPP+
E5 culture
serology
DPP+
E6 culture
serology
StatPak/MAPIA/DPP-
DPP+
E7 culture
serology
StatPak/MAPIA/DPP-
DPP+
E8 culture
serology
StatPak/MAPIA/DPP-
DPP+