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2 elephants at the captive-wild interface

3 **Authors:** Sarad Paudel^{1,2*}, Susan K. Mikota^{3*}, Jeewan Thapa^{4*}, Konstantin P. Lyashchenko⁵,
4 Kamal Prasad Gairhe⁶, Ishwari Prasad Dhakal⁷, Naresh Subedi⁸, Bhagwan Maharjan⁹, Suraj
5 Subedi⁸, Gretchen E. Kaufman¹⁰, Toshio Tsubota¹

6 **Author's affiliation**

7 ¹ Laboratory of Wildlife Biology and Medicine
8 Graduate School of Veterinary Medicine
9 Hokkaido University
10 Kita 18, Nishi 9, Kita-ku,
11 Sapporo 060-0818, Japan

12 ² Current Address
13 Department of Cell Physiology
14 Faculty of Medicine and Graduate School of Medicine
15 Hokkaido University
16 Kita 15, Nishi 7, Kita-ku
17 Sapporo, Hokkaido, 060-8638, Japan

18 ³ Elephant Care International
19 166 Limo View Lane
20 Hohenwald TN 38462, USA

21 ⁴ Hokkaido University Research Center for Zoonosis Control
22 Kita 20, Nishi 10, Kita-ku, Sapporo 001-0020, Japan

23 ⁵ Chembio Diagnostic Systems, Inc.
24 3661 Horseblock Road
25 Medford, NY 11763, USA

26 ⁶ Department of National Parks and Wildlife Conservation
27 Chitwan National Park, Chitwan, Nepal

28 ⁷ Agriculture and Forestry University
29 Rampur, Chitwan, Nepal

30 ⁸ National Trust for Nature Conservation
31 Khumaltar, Lalitpur, Nepal

32 ⁹ German Nepal Tuberculosis Project (GENETUP), Kalimati, Kathmandu, Nepal

¹⁰ Veterinary Initiative for Endangered Wildlife
1627 W. Main St., Suite 445, Bozeman, MT USA 59715

*Sarad Paudel, Susan K. Mikota and Jeewan Thapa contributed equally to this work

Corresponding author:

Toshio Tsubota
Laboratory of Wildlife Biology and Medicine
Graduate School of Veterinary Medicine
Hokkaido University
Kita 18, Nishi 9, Kita-ku,
Sapporo 060-0818, Japan
Phone: +81-11-706-5101; Fax: +81-11-706-5569
Email: tsubota@vetmed.hokudai.ac.jp

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

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

82

83 **Introduction**

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

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

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

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

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

116 **Materials and Methods**

117 **Study subjects and sample collection**

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

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

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

128 **Serology testing**

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

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

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

148 **Postmortem examination**

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

156 **Culture of trunk discharges and lung tissues**

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

161 **Results**

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

166 **Serological testing**

167 Two of six elephants tested by MAPIA in 2006 were seropositive (E2 and E3). E3 was
168 seropositive more than three years prior to diagnosis by culture. Elephants E6, E7, and E8 were
169 non-reactive on MAPIA in 2006, but showed positive reactivity on the DPP test and had

170 evidence of TB at postmortem examination 6-7 years later, suggesting that they were infected
171 and seroconverted after 2006.

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

175 All eight elephants reacted on the DPP VetTB assay at some point between 2006 and
176 2013, including two elephants that were tested using postmortem lung fluid. This is an off-label
177 use of the test but shows that TB antibodies are present in other body fluids.

178 **Postmortem**

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

182 **Culture**

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

190

191 **Discussion**

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

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

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

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

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

221 While *M. bovis* is a known threat to a variety of species in the wild such as Eurasian
222 badgers (*Meles meles*) (Drewe et al. 2010), and cape buffalo (*Syncerus caffer*) where in Kruger
223 National Park TB has spilled over to other mammalian species, including lions (Miller et al.
224 2012), it is only since 2013 that *M. tb* has been found in any wild species. A wild African
225 elephant that had been under human care was the first reported case although it is not clear
226 whether the mycobacterial species was confirmed (Obanda et al. 2013). Since then, four cases
227 with no history of captivity have been reported in wild Asian elephants in India (Zachariah et al.
228 2017; Chandranaik et al. 2017) and one additional case in Sri Lanka (Perera et al. 2014).

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

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

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

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

248

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256

257

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

370 *Footnote:* serology shown includes results of one or more tests during the time period indicated.

371 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 et 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)	<i>M. tb</i>	Aug 2009	Too old to treat
E4	F	56	coughing, weakness, trunk discharge	NA	R 2008, 2010	R 2010	P 2010 (n=1) <i>M.tb</i>	NA	2010; month unknown	<i>M.tb</i> isolated from trunk discharge; no PM or PM culture performed
E5	F	60	none	NA	NA	R 2009 (lung fluid)	NA	<i>M. tb</i>	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)	<i>M. tb</i>	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) <i>M. terrae</i> isolated from one sample	<i>M. tb</i>	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)	<i>M. tb</i>	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+