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Tuberculosis in captive Asian elephants (*Elephas maximus*) in Peninsular Malaysia

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SUMMARY

A cross-sectional study was conducted from 10 January to 9 April 2012, to determine the seroprevalence of tuberculosis (TB) of all captive Asian elephants and their handlers in six locations in Peninsular Malaysia. In addition, trunk-wash samples were examined for tubercle bacillus by culture and polymerase chain reaction (PCR). For 63 elephants and 149 elephant handlers, TB seroprevalence was estimated at 20·4% and 24·8%, respectively. From 151 trunk-wash samples, 24 acid-fast isolates were obtained, 23 of which were identified by *hsp*65-based sequencing as non-tuberculous mycobacteria. The *Mycobacterium tuberculosis*-specific PCR was positive in the trunk-wash samples from three elephants which were also seropositive. Conversely, the trunk wash from seven seropositive elephants were PCR negative. Hence, there was evidence of active and latent TB in the elephants and the high seroprevalence in the elephants and their handlers suggests frequent, close contact, two-way transmission between animals and humans within confined workplaces.

Key words: Captive Asian elephants, elephant handlers, *Mycobacterium tuberculosis*, non-tuberculous mycobacteria (NTM), Peninsular Malaysia.

INTRODUCTION

Tuberculosis (TB) is a serious chronic infection in humans and animals throughout the world. It affects a large variety of animal hosts including non-human primates, marine mammals like seals and sea lions, psittacine and other birds, domestic, captive and wild animals like cats, rats, cattle, sheep, goats, swine, deer, fox, badgers, moles and elephants [1–5]. Transmission between human and captive animals has occurred following close and frequent contact [6].

TB has been recognized as a disease of elephants for over 2000 years [7, 8]. However, naturally occurring TB has not been reported in wild elephants, suggesting that captive elephants could most likely have contracted the disease via contact with infected humans [1]. In captive elephants, the disease is primarily caused by *Mycobacterium tuberculosis*, although infection with *Mycobacterium bovis* has been recorded [9]. Asian elephants (*Elephas maximus*) are more frequently infected with TB compared to African elephants (*Loxodonta africana*) [10]. The difference in prevalence may reflect a closer association of Asian

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elephants with humans [1]. Rothschild & Laub [11] identified tuberculous lesions in 52% (59/113) of mastodon (*Mammut americanum*) skeletons, and implied that pandemic TB may be one of the probable causes of the mastodons' extinction. This discovery has alerted us to the need to protect living elephants from TB.

Transmission of TB from captive Asian elephants to other animals and humans has been described in several outbreaks [12–14]. Clinical signs in infected elephants are usually absent or only shown in the terminal stages of the chronic disease. Transmission of *M. tuberculosis* occurs by aerosolization of infectious respiratory droplets when the animals cough, trumpet or trunk spray, and is affected by the bacterial load, droplet size, duration of exposure, proximity to infected animals and the immune status of the exposed individual. TB can only be transmitted from elephants with active pulmonary disease following primary infection or after reactivation of latent infection [6].

The interest in elephant TB has been increasing over the past years due to its public health threat as well as increased concern for the healthcare and conservation of elephants. According to the World Conservation Union (IUCN red list 2006), the Asian elephant is an endangered species. There are probably about 1100–1200 Asian elephants in the wild and slightly more than 60 captive Asian elephants in Peninsular Malaysia. Although there are a number of recent reports of TB in captive Asian elephants from the USA [9], Sweden [14], Thailand [15], Nepal [16] and Sri Lanka [17], very little is known about its prevalence in Peninsular Malaysia. The aim of this study is to determine the prevalence of TB in captive Asian elephants in zoos and conservation centres in Peninsula Malaysia and to assess its potential risk of transmission between elephants and to elephant handlers. TB surveillance data would provide important information to determine the need for a strategy to prevent and control TB in elephants as well as a specific occupational health programme for elephant handlers and caregivers.

METHODS

Animal and human samples

We conducted a cross-sectional study between 10 January and 9 April 2012 to estimate the seroprevalence of *M. tuberculosis* infection in all 63 captive elephants in six locations (zoos and elephant conservation centres) across Peninsular Malaysia. Only 58 elephants that were approachable and allowed handling, without imposing significant risk to both elephants and handlers were sampled. At the same time, blood samples from elephant handlers (zoo keepers and workers, veterinary staff and *mahouts*) who gave informed consent for participation in the study were collected to estimate the extent of human exposure to TB. Only those with direct contact with elephants were screened. The Animal Care and Use Committee at the University Putra Malaysia and Department of Wildlife and National Parks Malaysia (DWNP) granted approval for the study on captive Asian elephants while the Medical Ethical Committee from the University Malaya Medical Centre granted approval for the study on elephant handlers.

Blood sampling and testing in elephants

We collected blood from the auricular vein behind the elephant's ear, using a 21-gauge needle butterfly catheter and a 10-ml syringe to draw blood into a plain tube. Blood was allowed to clot at room temperature and samples were shipped in an ice chest to the laboratory within 3–12 hours. Sera were collected by centrifuging the blood tubes at 600 ***g*** for 10 min at ambient temperature (25–30 °C) and then used in the ElephantTB Stat-Pak assay (ChemBio, USA) which contains a cocktail of recombinant antigens for rapid detection of antibodies to the *M. tuberculosis* complex in elephants [18].

Trunk-wash collection in elephants

Trunk-wash samples were collected by the elephant handlers under the supervision of the veterinarians in zoos and conservation centtes, using the 'triple sample method' [19]. A series of three trunk-wash samples was collected on separate days within a 1-week period. Each elephant was carefully restrained by its *mahouts* and 60 ml of sterile normal saline was instilled into a nostril using a 60-ml syringe. The trunk was lifted up and then lowered to collect the fluid in a sterile plastic bag. The wash was later split into two 25-ml aliquots and transferred into a 50-ml sterile Falcon tube™ (BD Biosciences, USA). One set of samples was taken for liquid culture (BACTEC MGIT960 system, BD Biosciences) and polymerase chain reaction (PCR) at the University of Malaya (UM), and the other set for culture on

Lowenstein–Jensen (LJ) agar (Oxoid, UK) at the University Putra Malaysia (UPM). A total of 154 trunk-wash samples were collected of which only 151 could be used.

Decontamination of trunk-wash samples

Prior to culture, trunk-wash samples were decontaminated using the modified Petroff method [20]. Briefly, the samples were centrifuged at 13 000 ***g*** for 15–25 min at 4 °C. The supernatant was discarded and 5 ml was retained in the tube to which 5 ml 2% NaOH was added. The mixture was incubated at 37 °C for 15 min. After incubation, 40 ml phosphate buffered saline (1 × PBS) was added and the mixture was centrifuged at 13 000 ***g*** for a further 15–25 min. The supernatant was then discarded, 1 ml 1 × PBS was added to resuspend the pellet and the sample was transferred into a labelled sterile bijou bottle.

Culture and microscopic identification

A loopful of each decontaminated sample was inoculated onto a LJ slant and incubated at 37 °C for up to 8 weeks. The cultures were examined daily for 7 days, then weekly for 7 weeks, during which time the growth rate and pigmentation of visible colonies were recorded. Ziehl–Neelsen staining was performed to look for acid-fast bacilli under a light microscope.

TB antigen rapid test (TiBilia™ TB, Genesis, China)

The TiBilia test, an immunochromatographic assay that detects the presence of MPB64 antigens exclusively found in the *M. tuberculosis* complex, was conducted for all acid-fast positive isolates. One loopful of each colony was suspended in 200 μl extraction buffer in a 1·5-ml tube. The mixture was vortexed and 100 μl was dropped into the sample well of the test device at room temperature. Results were read after 15 min and the presence of two red lines at the test and control areas indicated positive detection for *M. tuberculosis*, while a red line only at the control area was considered negative. Test results showing no red line or a red line at the test area only were considered invalid.

PCR detection of *M. tuberculosis* in trunk wash

DNA in the trunk wash was extracted and purified using DNA-sorb-B Nucleic Acid Extraction kit (AmpliSens Biotechnologies, Russia), according to the manufacturer's instructions. The purified DNA then served as a template for PCR amplification of the *M. tuberculosis* complex using the AmpliSens MBT-EPh PCR kit (AmpliSens Biotechnologies).

Identification of non-tuberculous mycobacterium (NTM) species

One loopful of an isolate on LJ medium was suspended in 0·2 ml sterile distilled water. The resulting suspension was boiled at 100 °C for 30 min and then centrifuged at 16 100 ***g*** for 2 min. The resulting supernatant was used for the subsequent *hsp*65-based PCR analysis, as described by McNabb *et al.* [21]. PCR amplicons were purified by the QIAquick PCR Purification kit (Qiagen, Germany) and sequenced by 1st BASE Laboratories (Malaysia). The resulting DNA sequences were aligned, using BLAST, against *hsp*65 locus sequences in a web-accessible database (http://msis.mycobacteria.info/). The most probable species of each isolate was identified based on the sequence similarity with reference strains and the expectation value (E value).

Blood sampling and testing of elephant handlers

Venous blood samples from the elephant handlers, caregivers and veterinary staff were collected by medical staff from the Ministry of Health, Malaysia, for testing with the QuantiFERON®-TB Gold In-Tube test (Cellestis Inc., Australia), which detects the release of interferon (IFN)-γ from TB-specific T lymphocytes [22]. Three millilitres of blood was drawn directly into three blood collection tubes, i.e. 1 ml each into a nil control tube with saline, TB antigen tube with a mixture of synthetic peptides representing ESAT-6, CFP-10 and TB7·7 test antigens, and a mitogen control tube containing phytohaemagglutinin. To ensure complete mixing of the blood with the tube contents, the tubes were shaken immediately after blood collection and again just before they were incubated at 37 °C, within 16 h of collection. After 16–24 h incubation, the tubes were centrifuged and the supernatant containing IFN-γ released from the antigen-stimulated T lymphocytes was harvested for testing by QuantiFERON-TB Gold ELISA. As recommended by the manufacturer, optical density readings >0·35 IU/ml were interpreted as indicative of latent or active TB infection, depending on clinical presentation.

Table 1. *TB Stat-Pak test results for captive Asian elephants by location*

Location	No. of elephants	No. of sera collected	No. of sera tested	No. of Elephant TB Stat-Pak positives	Seroprevalence (%)
A	27	27	27	7	25·9
B	8	8	8	2	25·0
C	8	6	5	0	0·0
D	9	9	6	1	16·7
E	3	3	2	0	0·0
F	8	3	1	0	0·0
Total	63	58	49	10	20·4

Data analysis

TB seroprevalence was estimated by the number of seropositives divided by the total number tested, and reported as a percentage (%). Statistical analysis was performed using Fisher's exact test from GraphPad InStat version 3 (GraphPad Inc., USA). A P value <0·05 was considered statistically significant.

RESULTS

Fifty-eight serum samples were obtained from 63 elephants in captivity, of which only 49 could be tested as the rest were haemolysed. Using the rapid Stat-Pak assay, 10/49 (20·4%) elephants tested were seropositive (Table 1). Herd prevalence ranged from 0% to 25·9% in the six study locations. Of 149 staff who had contact with elephants, the overall seroprevalence by QuantiFERON test was 24·8% (range 18·6–50%) (Table 2). Besides Malaysians, there were 19 foreign nationals among the elephant handlers. There was no significant difference in seropositivity (P=0·2537) between Malaysian (23·18%, 30/130) and foreign (36·8%, 7/19) workers. Neither was there any significant association with duration of employment (P> 0·9999 for duration ~1 year and P>0·9999 for duration ~5 years).

The TB detection results for trunk-wash samples are given in Table 3. Samples were only collected from five locations as the elephants in location F were not approachable. TB PCR was performed on all 10 seropositive elephants and 12 of the seronegatives animals. Of these 22 elephants, three (13·6%) were positive in both tests and therefore considered to have laboratory evidence of active TB. Eight elephants (with negative serology and PCR) were probably not infected. Seven (seropositive but PCR-negative) were considered to have latent infection and the remaining four (seronegative but PCR-positive) could be false-positive PCR or false-negative serology results.

Table 2. *QuantiFERON test results of elephant handlers by location*

Location	No. of human samples tested	No. of QuantiFERON test positives	Prevalence (%)
A	70	13	18·6
B	21	5	23·8
C	20	5	25·0
D	12	6	50·0
E	18	6	33·3
F	8	2	25·0
Total	149	37	24·8

All trunk-wash samples were cultured; none grew *M. tuberculosis*. Most of the liquid cultures were heavily contaminated by non acid-fast bacteria and fungi, despite prior decontamination and the incorporation of antibiotics (BBL, MGIT PANTA; BD Biosciences) into the MGIT culture medium. However, on LJ slants it was possible to obtain pure subcultures of acid-fast bacteria, but 23/24 positive cultures turned out to be NTM species, identified by a negative TiBilia test followed by *hsp*65gene amplification and sequence alignment with reference NTM species. *M. arupense* and *M. colombiense* made up 50% of the NTM species recovered (Table 4).

DISCUSSION

Many techniques have been used for the detection of TB in elephants but few have been reported to be entirely satisfactory when used alone. A combination of diagnostic assays is often required [15]. The Chembio TB Stat-Pak assay used in this study is

Table 3. *Culture and TB PCR results of elephant trunk washes by location*

Location	No. of elephants	No. of trunk washes collected	No. of acid-fast positive cultures	No. of elephants TB PCR positive
A	27	78	13	0
B	8	24	4	2
C	8	18	2	0
D	9	27	4	3
E	3	4	1	2
F	8	0	0	0
Total	63	151	24*	7†

PCR, Polymerase chain reaction.
* From 55 elephants.
† From 22 elephants tested.

Table 4. *Non-tuberculous mycobacteria (NTM) identification by hsp65 sequencing*

Most probable species	No. of isolates
M. arupense	7
M. colombiense	5
M. intracellulare	2
M. asiaticum	2
M. mantenii	2
M. fortuitum	2
M. gilvum	1
M. hiberniae	1
M. kumamotonense	1
Total	23*

* Of 24 acid-fast bacilli-positive cultures, 23 were NTM species. The remaining isolate was identified as *Nocardia nova*.

licensed by the U.S. Department of Agriculture as a screening test for TB in elephants. The sensitivity and specificity of this test for the detection of anti-*M. tuberculosis* complex antibodies have been reported to be 100% and 95%, respectively [23]. However, other workers have noted an inadequacy of the Stat-Pak assay for the unequivocal identification of TB-infected animals [9, 15]. Our results also showed poor correlation between serology and trunk-wash culture and PCR. In humans, serological results are affected by the phase of TB infection and the immune competence of the host. A positive serological result in the absence of clinical features and *M. tuberculosis* detection is usually interpreted as indicative of latent infection, while negative serology in the presence of positive TB culture or PCR can be explained by immunological anergy. It is not known whether the same interpretations are applicable in elephants. With trunk-wash tests, sensitivity has been reported to be poor [18, 23] and affected by collection and processing methods as well as the degree of contamination in the samples collected. The PCR assay we used is designed for a wide range of human specimen types but has not been adequately evaluated for elephant respiratory samples. Hence, although the combined use of both the Stat-Pak assay and TB PCR did provide some evidence for active and latent TB infection in our captive elephants, continued monitoring of the health of elephants, particularly those seropositive, and repeated examination of trunk washes are required to confirm TB infection in elephants. Nevertheless, there is sufficient indication that there exists a sizable reservoir of silent infection in the elephants that would maintain continued transmission if not controlled.

The QuantiFERON test has been well established for the diagnosis of latent TB in humans and is often used as a supplementary test to aid the diagnosis of active TB. None of the 37 staff who tested positive in this study had TB-like symptoms. All 13 seropositive individuals from location A were examined by a chest physician and found to have no physical or radiological signs. Only one gave a history of recent contact with a known case of TB. The 24·8% seroprevalence is probably entirely due to latent infection but it is substantially higher than the seroprevalence previously obtained (authors' unpublished data) for asymptomatic university lecturers and students (4%), general laboratory staff (6%) and staff working in a TB diagnostic laboratory (12·5%). The overall 20·4% seroprevalence in our elephants is comparable to reports of 20% from Nepal [16] and 12–25% from India (25% of elephants in temples *vs.* 12–15% in

other elephant groups) [16]. This finding is rather unexpected as the human TB incidence in Malaysia (3-year average of 83/100 000 population from 2008 to 2010) is substantially lower than incidences in Nepal (163/100 000 population) and India (190/100 000 population) [24]. Hence, the high seroprevalence in elephants and their handlers in this study could be the result of frequent, close contact, two-way transmission between animals and humans within confined workplaces.

The isolation of NTM from trunk wash illustrates the ubiquitous presence of these environmental bacteria. Many of the species recovered are potential animal and human pathogens. *M. avium* has been reported to be the most frequently isolated mycobacterial species from trunk washes in the USA [9]. Similarly, in this study, among the most frequently isolated NTM were two members of the *M. avium* complex, *M. colombiense* (a new sequevar of *M. avium* [25]) and *M. intracellulare. M. arupense,* the other common isolate, is associated with human respiratory infections and has been isolated from various domestic and wild animals [26]. The role of these NTM species as elephant pathogens requires further investigation.

There is a paucity of information of TB in elephants particularly in Asia. To the best of our knowledge, this study is the first to look at the extent of TB infection in captive Asian elephants in Peninsular Malaysia. Our findings could contribute to the development of a long-term surveillance and healthcare programme for this endangered species. From the conservation point of view, the implication of potential transmission and spread to wild elephant populations cannot be underestimated, e.g. if TB-infected elephants are used during rescue and release operations [27]. Just as important is the prevention of elephant–human transmission in zoos and conservation centres. Elephant handlers need to be aware of the risk of TB acquisition from infected animals and be educated with regard to infection control measures. On the other hand, as elephants become infected by humans with open TB, there must be workplace policies (e.g. pre-employment screening and annual chest X-rays) to ensure that elephant handlers are free from active TB. Hence, a One Health approach [16] involving both animal and human health sectors should be undertaken to develop a comprehensive prevention, treatment and prophylaxis control strategy to protect the elephants and their caregivers from TB.

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DECLARATION OF INTEREST

None.

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