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COMMUNICATION

Sero-diagnosis of tuberculosis in elephants in Maharashtra, India

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Abstract: Tuberculosis is a highly contagious zoonotic disease caused by *Mycobacterium* spp. A study was conducted to detect the presence of *Mycobacterium* in captive elephants. A total of 15 captive elephants were screened from various regions in Maharashtra. The blood and serum samples collected were subjected to rapid test kit, BacT/ALERT 3D system, Ziehl-Neelsen (ZN) staining and PCR. All the samples were found seronegative using rapid test kit and whole blood PCR. Whereas, all samples were signalled culture positive in BacT/ALERT 3D system which were further subjected to PCR, only one amplicon was produced of 176bp of RD4 gene (*Mycobacterium bovis*) and no acid-fast organism was detected upon ZN. Due to the atypical nature of this organism, diagnosis of this disease in elephants using various tests is complicated unlike the diagnostic tests that are validated in domestic animals. Therefore, many tests have sub-optimal sensitivity and specificity in elephants. As TB is a zoonotic disease, transmission can occur between human-livestock-elephants interface. Therefore, the zoos and state forest authority should inculcate a protocol of periodic TB screening for Mahouts and elephants in captivity along with protocol of elephant-visitor interaction, thus helping in conservation of this endangered species in India.

Keywords: Elephants, mycobacterium, serodiagnosis, Tuberculosis.

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INTRODUCTION

Elephants are the largest terrestrial mammals on the earth. Elephants belong to the family Elephantidae in animal kingdom. Two genera *Elephas* and *Loxodonta* and three species are present today – the Asian Elephant *Elephas maximus*, the African Bush Elephant *Loxodonta africana*, and the African Forest Elephant *Loxodonta cyclotis*.

Currently, a population of 27,312 elephants has been estimated from 23 states in India (Project Elephant Division, Government of India, 2017). In past decades, the population of elephants has drastically been reduced and since 1986, the Asian Elephant has been listed as 'Endangered' species on the IUCN Red List, as the wild population has declined by at least 50% (Choudhury et al. 2008). The Asian Elephant is placed in Schedule I and Part I of Indian Wildlife Protection Act (1972); conferring it the highest level of protection.

Tuberculosis is a highly contagious zoonotic disease in animals as well as humans. It is caused by highly pathogenic bacteria of *Mycobacterium tuberculosis* complex (MTBC) which are *M. tuberculosis, M. bovis*, and *M. canetti*. The *M. tuberculosis* and *M. bovis* are most pathogenic. Tuberculosis (TB) in elephants was first observed more than 2,000 years ago by ancient Ayurvedic physicians in Ceylon (Iyer 1937; McGaughey 1961). Transmission between human and captive animals has occurred following close and frequent contact (Kathleen et al. 2002). More frequent reporting of this disease occurs in Asian Elephants than in African Elephants may be due to closer human contact related to their use for performances, rides and in temple rituals. *Mycobacterium tuberculosis* is the predominating disease-causing agent in elephants, although TB cases have been caused by *M. bovis* (Mikota 2008). The reservoirs for *M. tuberculosis* and *M. bovis* are infected human and cattle (Hirsch 2004).

Elephants with tuberculosis infection show clinical signs like weight loss, wasting and weakness, coughing or dyspnoea have been reported but appear to be uncommon. Exercise intolerance may be observed in working elephants (Mikota 2008). In some cases, ventral oedema has been reported, but other pathologic factors could be the initiating cause (Seneviratna et al. 1966). Majority of times elephants infected with TB do not have any clinical signs. In some cases, elephants manifest symptoms only in advance stage of disease or may not be diagnosed until necropsy (Paudel & Tsubota 2016).

The study presents the clinical, serological, and culture data from 15 elephants present in captivity thus helping to diagnose and decrease TB risk to these wild animals.

MATERIALS AND METHODS

Study animals and sample collection

Blood and serum samples were collected from the 15 elephants in captivity of Forest Camp areas of Gadchiroli (19.4290° N, 80.0563° E), Pune Zoo (18.452^{0}N, 73.865^{0}E), Mumbai Zoo (18.978^{0}N, 72.835^{0}E), Shegaon temple (20.789^{0}N, 76.701^{0}E) in Maharashtra. The elephants were included in the study irrespective of their health status, age, sex or habitat.

Serological testing

The Wild TB alert kit is a lateral flow chromatographic immunoassay for the detection of antibodies of mycobacterium tuberculosis complex antigenserum, plasma and whole blood of elephants. This kit contents a unique cocktail of tuberculosis specific recombinant proteins (ESAT-6, CFP-10, MPB83, MPB70) and crude protein impregnated on nitrocellulose membrane housed in a disposable plastic cassette. After adding sample to the well followed by addition of diluent they travel through the membrane by capillary action. If antibodies are present, they bind to the antigen and a red colour band is observed in test area.

BacT/ALERT 3D system

BacT/ALERT 3D system is an automated microbial detection system which offers microbiological culture of blood. This mycobacteria detection systems utilize a colorimetric sensor and reflected light to monitor the presence and production of carbon dioxide (CO_2) dissolved in the culture medium. BacT/ALERT MB are disposable culture bottles with a removable closure contain 10 ml of media and an internal sensor that detects carbon dioxide as an indicator of microbial growth. The media formulation consists of: Middlebrook 7H9 Broth (0.47% w/v), Pancreatic Digest of Casein (0.1% w/v), Bovine Serum Albumin (1.0% w/v), Catalyse (48 µ/ml), in purified water. Bottle reflectance is monitored and recorded by the instrument every 10 minutes. The growth curve enters lag phase then the bottle is flagged positive. At the time of detection, approximate colony forming units (CFUs) are 106–107 per ml.

Ziehl-Neelsen/Acid Fast staining

Bacterial culture smear was prepared from samples indicated positive in BacT/ALERT 3D system on clean

and grease free slide, using standard protocol of Ziehl-Neelsen staining kit (Hi-Media Pvt. Ltd, India).

PCR detection of mycobacterium

DNA was extracted from blood samples and samples signaled positive in BacT/ALERT 3D system of 15 elephants using the extraction protocol described by Samrook et al. 1989 and Tissue Genomic DNA Extraction Mini Kit (FAVORGEN Biotech Corp, Taiwan). The extracted DNA was subjected to PCR by using the standard primer RD4 F 5'-AATGGTTTGGTCATGACGCCTTC-3'; R 5'-CCCGTAGCGTTACTGAGAAATTGC-3' and RD1 F 5'-CCCTTTCTCGTGTTTATAGTTTGA-3' R 5'-GCCATATCGTCCGGAGCTT-3' which was amplified 176 and 110 bp of *Mycobacterium tuberculosis* and *Mycobacterium bovis*. The PCR reaction was carried out at 94°C for 10 minutes followed by 35 cycles of denaturation at 94°C for 1 minute, annealing at 60°C for 30 seconds and extension at 72°C for 1 minute, with final extension at 72°C for 10 minutes. The PCR products were analysed by electrophoresis in 1.5% agarose gel at100 V for 45 minutes and documented. Amplicon of size 176bp and 110bp is specific for Mycobacterium genus.

RESULTS

The Table 1 shows the results of various diagnostic tests used for diagnosis of mycobacterium in elephants. The serum samples collected from the 15 elephants were seronegative by the rapid test kit as no coloured band was observed in the test area of the rapid test kit (Image 1). All the 15 samples were detected positive by the BacT/ALERT 3D system in 6 mean days. These samples were further subjected to ZN staining, no sample detected the presence of acid fast bacilli (Amer et al. 2016; Bapat et al. 2017) (Image 2). Isolates of DNA extracted from the blood samples of these 15 elephants were subjected to PCR which did not produce specific amplicon of 176bp and 110bp RD4 and RD1 gene. Similarly, the DNA isolates from the BacT/ALERT culture system did not produce amplicon of 176 and 110 bp but one isolate produced amplicon of 176bp of RD4 of targeted gene indicating presence of *Mycobacterium bovis* (BCG) (Bapat et al. 2017) as illustrated in Image 3 and 4.

DISCUSSION

Tuberculosis is a highly contagious zoonotic disease with high incidence and prevalence in human, domestic and wild animals of developing countries. Tuberculosis infection in captive elephants is ongoing and complex problem with respect to their conservation. Due to atypical nature of the mycobacteria that causes diseases, the diagnosis is rather complicated, apart from the fact that many diagnostic tests are developed for domestic species however, those are not validated for wild animals. Therefore, many tests have sub-optimal specificity and sensitivity.

Table 1. Overall results of test applied (n= 15).

Elephant No.	BacT/ALERT	ZN Staining	Blood PCR	BacT/ALERT + ve PCR	Rapid test
(E1)	Positive	Negative	Negative	Negative	Negative
(E2)	Positive	Negative	Negative	Negative	Negative
(E3)	Positive	Negative	Negative	Negative	Negative
(E4)	Positive	Negative	Negative	Negative	Negative
(E5)	Positive	Negative	Negative	Negative	Negative
(E6)	Positive	Negative	Negative	Negative	Negative
(E7)	Positive	Negative	Negative	Negative	Negative
(E8)	Positive	Negative	Negative	**Positive**	Negative
(E9)	Positive	Negative	Negative	Negative	Negative
(E10)	Positive	Negative	Negative	Negative	Negative
(E11)	Positive	Negative	Negative	Negative	Negative
(E12)	Positive	Negative	Negative	Negative	Negative
(E13)	Positive	Negative	Negative	Negative	Negative
(E14)	Positive	Negative	Negative	Negative	Negative
(E15)	Positive	Negative	Negative	Negative	Negative

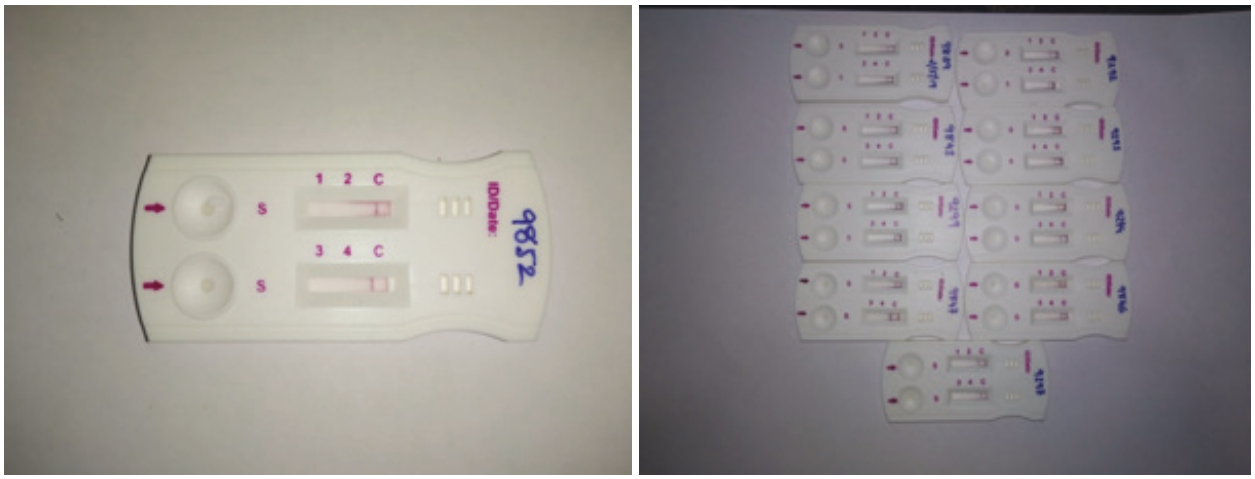

Image 1. Results of rapid TB test kit in elephants screened for tuberculosis.

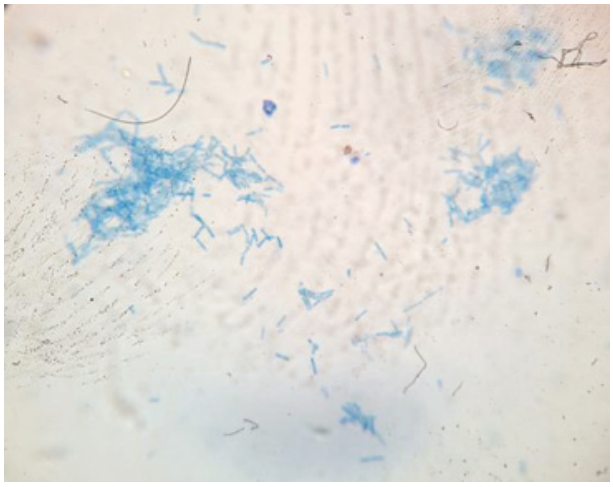

Image 2. Non-acid fast bacilli under microscope (100x) in elephants screened for tuberculosis.

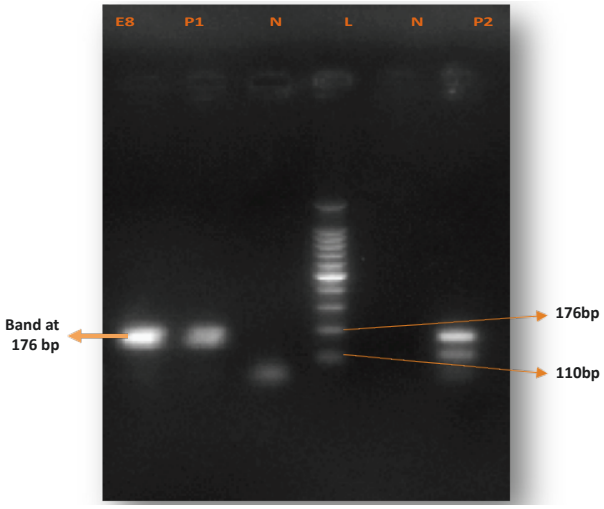

Image 3. PCR pattern of RD4 and RD1 gene at 176bp and 110bp of BacT/ALERT tuberculosis positive sample.
Lane E8: positive sample showing band at 176bp of RD4 gene, Lane P1: positive control (*M. bovis*), Lane P2: Postive control (*M. tuberculosis & M. bovis*), Lane N: negative control, Lane L: DNA ladder 100 bp.

The major problem to designate a perfect test among available tests for diagnosis of tuberculosis, which are most accurate for elephants, giving veterinarians a standardized method, which will allow them to make preventive measures and treatment protocols; thus, helping in conservation of endangered species like elephant.

These samples were subjected to diagnostic tests like BacT/ALERT 3D system, ZN staining, PCR, Rapid TB test kit. All 15 samples were signalled positive by BacT/ALERT 3D system. This test is not yet used and validated in animals, like in humans. This was the first time when the test was used in detection of TB in wild animals. Therefore, the specificity still remains a question. On the other hand, other tests like ZN staining, Rapid TB test kit and blood PCR did not detect any mycobacteria in the samples.

Molecular detection (duplex PCR) of the samples that signalled positive in BacT/ALERT 3D system was carried out using RD4 and RD1 gene primer with amplicon size of 176bp and 110bp respectively as described by Bapat et al. (2017). Only one sample was positive detecting the presence of *M. bovis* (BCG) at 176bp of RD4 gene.

During the study it was not possible to calculate the specificity of various diagnostic tests used. Development and use of new and more species specific diagnostic methods are needed at the moment, as it will help in early and accurate diagnosis that might permit early application of preventive measures and will ensure

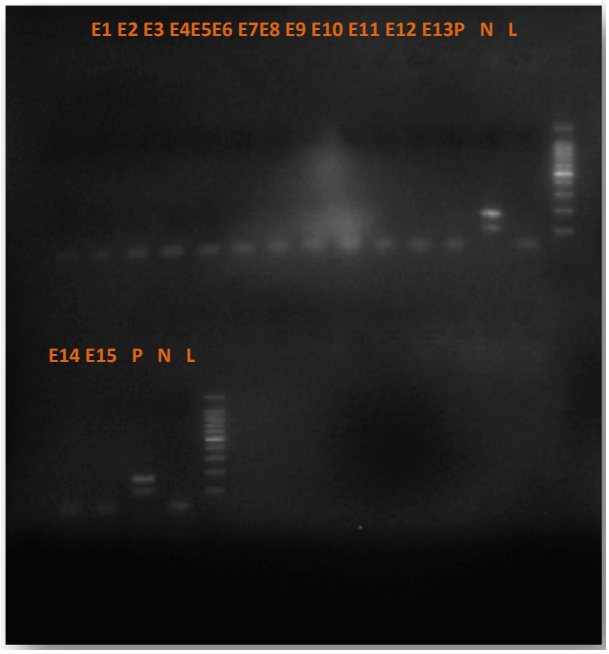

Image 4. PCR pattern of RD4 and RD1 gene at 176bp and 110bp of blood samples
Lane E1-15: negative elephant DNA isolates, Lane P: Positive control (*M. tuberculosis* & *M. bovis*), Lane N: negative control, Lane L: DNA ladder 100bp.

Specimen/DNA Museum Information:
Specimen: Blood.
Museum: Niche Area of Excellence, Centre for Zoonoses, Indian Council of Agriculture Research (Central India), Nagpur Veterinary College, Nagpur.
Voucher Number: NAE9299.

safety of endangered species as well as human staff involved. Moreover, this mycobacterial disease requires long term surveillance plans in order to be effective, as this organism has prolonged incubation and latency.

Although, the reported case of TB in elephant in present study was caused by *M. bovis* (BCG) which is vaccine strain, its species predilection is still unidentified. Moreover, this animal should be screened multiple times over the period of time to confirm the disease. Cultural isolation of mycobacterium is currently the only gold standard test for TB diagnosis in elephants, but ancillary tests like PCR, BacT/Alert 3D system, rapid TB test kit etc. may be useful. The molecular method (PCR) used in diagnosis of mycobacterium in present study is not a confirmatory test due to its possibility of cross contamination (false positive) and inability to determine the pathogenicity of the organism. As this is a zoonotic disease, transmission of TB can occur between humans, livestock and elephants. Elephants are at risk of contracting TB from infected human (Mahouts). Therefore, Mahouts (handlers) and elephants should undergo periodic TB screening to minimize the risk of animals' health. Zoos and forest elephant camp areas should be encouraged to incorporate protocol for elephant-visitor interactions and periodic screening of animals for tuberculosis.

This study highlights the potential usefulness and efficacy of ante-mortem diagnostic methods. Use of multiple tests helps to achieve high possibility (sensitivity) of tuberculosis detection in elephants rather than using single test; however, it is important to evaluate and validate the test regime and will require addition of more animals in to the study; expectantly allowing in better understanding of tuberculosis in elephants, thus contributing to undertake control measures by state forest department and zoo authorities for conservation of this endangered species.

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Author contributions: Dr. Utkarsh Rajhans, designed and conducted study on Sero-diagnosis of tuberculosis in elephants in Maharashtra, India. Dr. Gayatri Wankhede and Dr. Balaji Ambore helped in coordinated and guided in the research and manuscript writeup. Dr. Sandeep Chaudhari, Dr. Vitthal Dhaygude and Dr. Chhaya Sonekar designed, performed and analyzed the diagnostic procedures and data. The manuscript was written by Dr. Utkarsh Rajhans and commented by all authors.

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