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Pathological and molecular characterization of *Mycobacterium tuberculosis* complex in necropsied tissues of sambar deer (*Rusa unicolor*) and nilgai (*Boselaphus tragocamelus*) from Assam state, India

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Abstract

Bovine tuberculosis is a chronic bacterial disease primarily caused by *Mycobacterium bovis*, a member of the *Mycobacterium tuberculosis* complex (MTBC), with significant zoonotic implications. This study aimed to detect MTBC in wildlife species, specifically nilgai (*Boselaphus tragocamelus*) and sambar deer (*Rusa unicolor*), using gross pathology, histopathology, acid-fast staining, and molecular confirmation. Necropsied tissue samples were collected during post-mortem examination of a nilgai and a sambar deer from the Assam State Zoo, Guwahati, India. Macroscopically, multiple granulomatous tubercles of varying sizes were observed in the lungs and liver, with creamy white caseous material marked upon sectioning. Ziehl-Neelsen staining of the tissue smears from granulomatous lesions confirmed the presence of acid-fast bacilli. Microscopic examination of tuberculosis granulomas revealed a central necrotic mass surrounded by inflammatory cell infiltration, including Langerhans-type giant cells. Molecular confirmation of MTBC infection was achieved by amplifying *hsp65* and *IS1081* in tissue samples, further validated by Basic Local Alignment Search Tool for nucleotide analysis following Sanger dideoxy sequencing. In conclusion, this study confirmed the presence of tuberculosis in these wildlife species through an integrated approach combining pathology, microbiology, and molecular diagnostics, highlighting the need to understand pathogen entry into the herd and prevent potential spillover.

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Introduction

Bovine tuberculosis is a chronic bacterial zoonotic disease primarily caused by *Mycobacterium bovis*, a member of the *M. tuberculosis* complex (MTBC). Identifying *M. bovis* infection remains a challenge in both veterinary and medical sciences. The MTBC includes several species, such as *M. tuberculosis*, *M. bovis*, *M. orygis*, *M. africanum*, *M. microti*, *M. mungi*, *M. canetti*, *M. caprae*, and *M. pinnipedii*, all of which are responsible for the re-emerging chronic infectious tuberculosis.[1,2] Among these, *M. tuberculosis* and *M. bovis* are the primary pathogens, predominantly infecting humans and animals, respectively. The *M. bovis* is a zoonotic pathogen with a broad host range, affecting over 40 species of free-ranging wild animals, including deer species, and causing disease conditions, such as pneumonia, weight loss, and mortality.[3,4]

Various Southeast Asian countries have published reports of tuberculosis in wildlife, primarily in Asian elephants. It is commonly known that cattle serve as a primary reservoir for *M. bovis*, but other species, including humans, goats, pigs, buffaloes, dogs, monkeys, badgers, deer, possums, and bisons, are also susceptible hosts.[5] Zoonotic tuberculosis spreading to human is a threat for public health and has been documented in both domestic and wild animals globally.[6,7] Deer are among the wild animals that can contract MTBC and spread tuberculosis to livestock and other wild animals. Transmission of these diseases can spread orally from sick animals to humans or animals and by direct contact through aerosol.[8,9]

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The present study focused on detection of MTBC in a sambar deer (*Rusa unicolor*) and a nilgai (*Boselaphus tragocamelus*) through pathological and molecular investigations. Data regarding tuberculosis cases in these species in the Assam state, north-east region of India, remain undocumented due to the lack of surveillance programs for tuberculosis in wildlife. The sambar deer, native to the Indian sub-continent, South China, and Southeast Asia, has been categorized as a vulnerable species on the International Union for Conservation of Nature red list since 2008. The nilgai, also known as the blue cow, is the largest antelope in Asia and is widely distributed across the northern Indian sub-continent.

It has been demonstrated that an important obstacle for tuberculosis eradication in cattle is the wildlife implication.[10] In India, diagnosis of tuberculosis in animals is based on tuberculin test, routine post-mortem examination at slaughter house, and histopathological examination of tissues in the laboratory. Mycobacteria are fastidious and take long time for culture. So, many suspected cases of tuberculosis may be missed owing to non-availability of laboratories specialized in mycobacteria. To achieve a diagnosis in time as early as, in particular field samples, the use of PCR applied to direct tissue samples appears to be a promising technology that would be of practical use in making conclusive diagnoses.[11] There are some published reports of the application of PCR on direct tissue samples that had characteristic lesions and acid-fast organisms. The present study reports based on the application of PCR test using *hsp65* and *IS1081* insertion sequence on direct tissues from deer and nilgai that died of suspected tuberculosis along with aimed at studying necropsy lesions and histopathological findings of tuberculosis due to the MTBC infection. Therefore, regular livestock and wildlife screening will help to prevent zoonotic tuberculosis transmission to other animals and human.

Materials and Methods

Sampling location. This study was conducted on two animals, including a sambar deer (*R. unicolor*) and a nilgai (*B. tragocamelus*) that died suddenly at the Assam State Zoological and Botanical Garden, located in Guwahati, India (latitude: 26.16352and longitude: 91.78073). The zoo houses a total population of 138 sambar deer and seven nilgai, being maintained separately. Ethical clearance for the study was obtained from the Office of the Forest Veterinary Officer, Assam State Zoo Division, Government of Assam, India (File No. FVO/LR/2023-24/11 and FVO/LR/2023-24/100).

History. The nilgai exhibited signs of anorexia and lethargy before succumbing to sudden death, with noticeable drooling of watery fluid from the mouth. Prior to death, respiratory symptoms, like dyspnea and irregular coughing, were observed. In contrast, the sambar deer had a persistent cough and was under treatment at the time of death. Following the death of these animals in 2023, a necropsy was conducted, and the findings were documented by the attending veterinarian.

Sample collection and processing. A total of four clinically suspected tissue samples, including lungs and liver from both animals, were collected aseptically in plastic tissue container procured from Himedia, Thane, India. The samples were divided into two sets as follows: One was fixed in 10.00% buffered formalin for histopathological analysis, while the other was preserved fresh for bacteriological and molecular detections. The presence and type of lesions indicative of tuberculosis were characterized through microscopic examination. For histopathological analysis, paraffin blocks were prepared from formalin-fixed tissue samples, sectioned at a 5.00 µm thickness, stained with Hematoxylin and Eosin, and examined under a light microscope.[12] The presence of acid-alcohol-resistant bacilli was confirmed by microscopic examination of Ziehl-Neelsen-stained slides, following the standard method.[13]

Molecular identification. For molecular identification, DNA was extracted from tissue samples using Qiagen DNA extraction kit (Qiagen, Maryland, USA) following the manufacturer's protocol, and its quantification was performed using a NanoDrop spectro-photometer (Thermo Scientific, Waltham, USA). To detect MTBC in tissue samples, three sets of reference-based primers were used, including *Mycobacterium* genus-specific *hsp65* gene, the MTBC-specific *IS1081* sequence, and the *M. bovis*-specific 500 bp fragment.[14-16] The PCR amplification was carried out under the following conditions: An initial denaturation at 95.00 ˚C for 4 min, followed by 35 cycles of denaturation at 95.00 ˚C for 30 sec, annealing at 59.00, 69.00, and 59.00 ˚C for the respective primers for 30 sec, extension at 72.00 ºC for 35 sec, and a final extension at 72.00 ˚C for 7 min. The reactions were performed in thin-walled 200 µL PCR tubes using a total reaction volume of 20.00 µL, included 10.00 µL of DreamTaq Green PCR Master Mix (2.00 X; Thermo Scientific), containing DreamTaq DNA Polymerase, dNTPs, $MgCl_2$, an optimized buffer, 2.00 µL of template DNA, 1.00 µL of each primer (10.00 pmol), and 6.00 µL of nuclease-free water. Optimization was performed for each primer, and both positive (*M. bovis* AN5) and negative controls were included in every experiment to ensure the reliability of the results.

Results

The nilgai appeared grossly normal and had a fair body condition, whereas the sambar deer was in poor body condition. External examination of both animals revealed congested nasal discharge along with slightly swollen

mandibular and pre-scapular lymph nodes. Gross pathological changes were systematically recorded in various organs (Figs. 1 and 2). Both animals exhibited classical tuberculosis lesions, including small granulomatous tubercles of varying sizes and numbers in the lung parenchyma, and severe consolidation in lungs with gray and red hepatizations was also observed. Upon dissection, the granulomatous nodules contained creamy white caseous material. Similar encapsulated nodules of varying diameters were also observed in the liver, with yellowish-white caseous exudates oozing out upon incision, and also outside the liver. Externally, in liver and heart, hyperemic lesions were observed and the pericardial sacs of both animals were filled with dark-colored fluid.

Microscopic examination of the tuberculosis granulomas revealed a central necrotic mass surrounded by inflammatory cell infiltration, primarily composed of lymphocytes, macrophages, and a few plasma cells. Clear hemorrhagic areas were observed around the necrotic zones. Additionally, Langerhans-type giant cells surrounded by proliferating fibrous connective tissue were recorded under the microscope (Fig. 3). Ziehl-Neelsen staining of the tissue smears prepared from granulomatous lesions confirmed the presence of acid-alcohol-resistant bacilli (Fig. 4).

All four tissue samples from the sambar deer and nilgai tested positive for *Mycobacterium*, amplifying a 441 bp product specific to the *hsp65* gene (Fig. 5). Further confirmation as MTBC was achieved through amplification of the 248 bp of insertion sequence *IS1081* using PCR (Fig. 5). Additionally, all MTBC-positive samples amplified a 500 bp fragment of the *Mycobacterium* genome, indicating the possible presence of *M. bovis*. The PCR-amplified products were subjected to Sanger sequencing from both ends (forward and reverse), and a consensus sequence was generated using BioEdit® Software (version 10.0; Ibis Therapeutics, Carlsbad, USA). The consensus sequence was then compared with existing sequences in the National Center for Biotechnology Information database using a Basic Local Alignment Search Tool for nucleotide (nBLAST) search. The amplified *IS1081* sequence was submitted to National Center for Biotechnology Information GenBank with accession numbers of OR900192 (nilgai) and OR900193 (sambar deer).

Fig. 1. Gross pathological changes in a sambar deer. **A)** Photograph of emaciated sambar deer prior to necropsy; **B)** Cut section on lungs exhibiting creamy white caseous material; **C)** Accumulation of dark-colored fluid in pericardial sac (arrow); **D)** Enlargement of liver with hyperemic lesions (arrows).

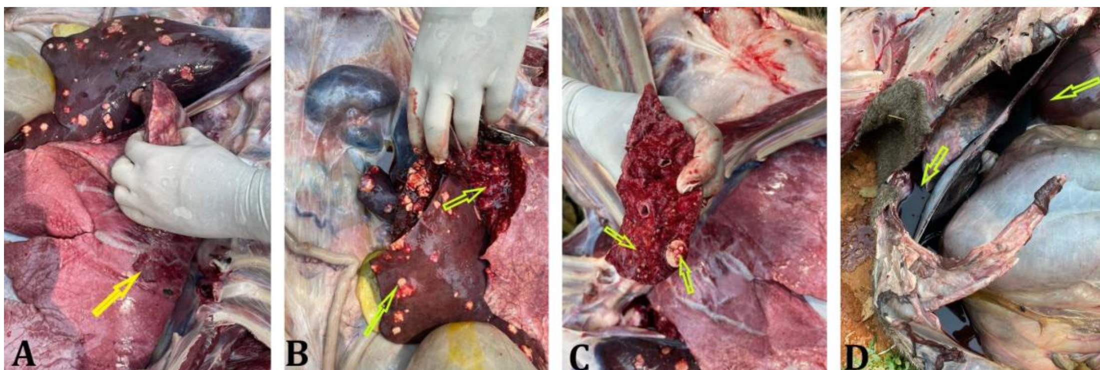

Fig. 2. Gross pathological changes in a nilgai. **A)** Red and grey hepatizations in the lungs (arrow); **B)** Nodule formation with white-yellowish pus in liver and lungs (arrows); **C)** Miliary to large tuberculous nodule formation with grey and red hepatizations in lungs (arrows); **D)** Hemorrhagic lesions in the heart with dark fluid accumulation in the pericardial sac (arrows).

Both sequences showed 99.00% similarity with *M. tuberculosis* strain MTb-Oman-321528 chromosome, complete genome.

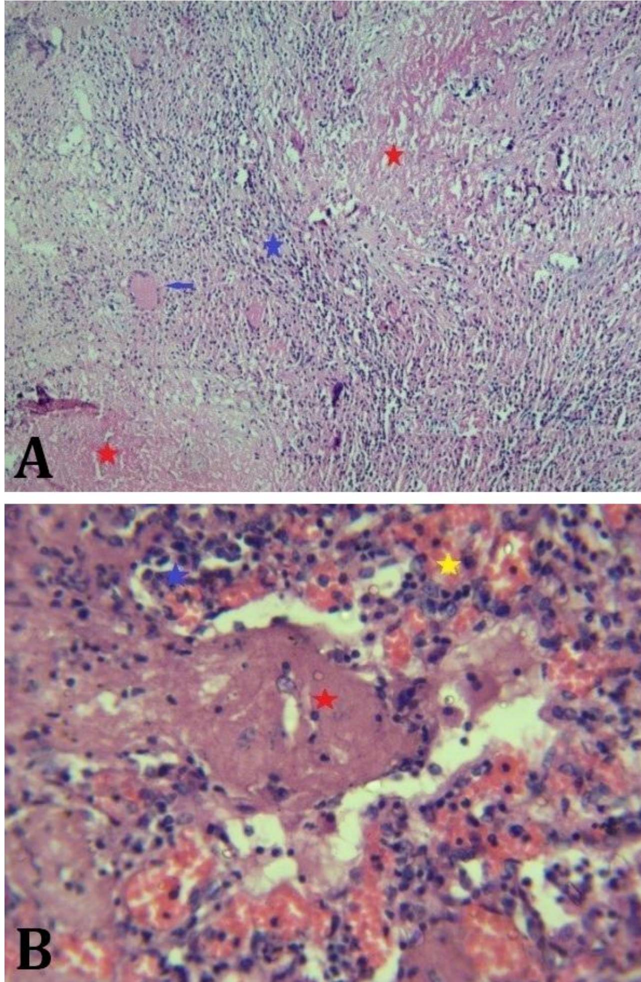

Fig. 3. A) and **B)** Histopathology of lung of nilgai showing coagulative necrosis (red asterisks), infiltration of inflammatory cells, mainly mononuclear ones (blue asterisks), giant cell (blue arrow), and hemorrhage (yellow asterisk) are evident (Hematoxylin and Eosin staining; 100× and 400×, **A** and **B**, respectively).

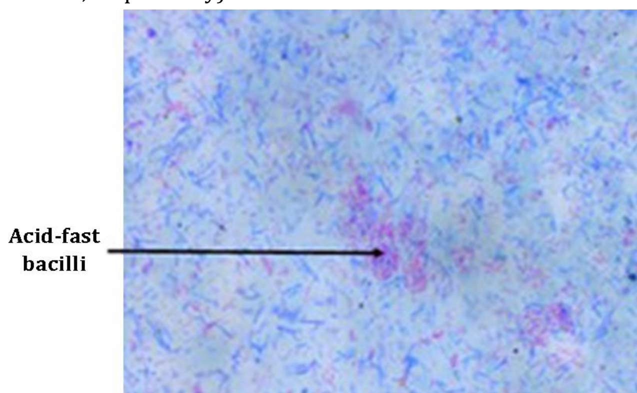

Fig. 4. Ziehl-Neelsen staining of the lung tissue smear. Presence of rod-shaped, reddish-pink acid-fast bacilli can be seen (400×).

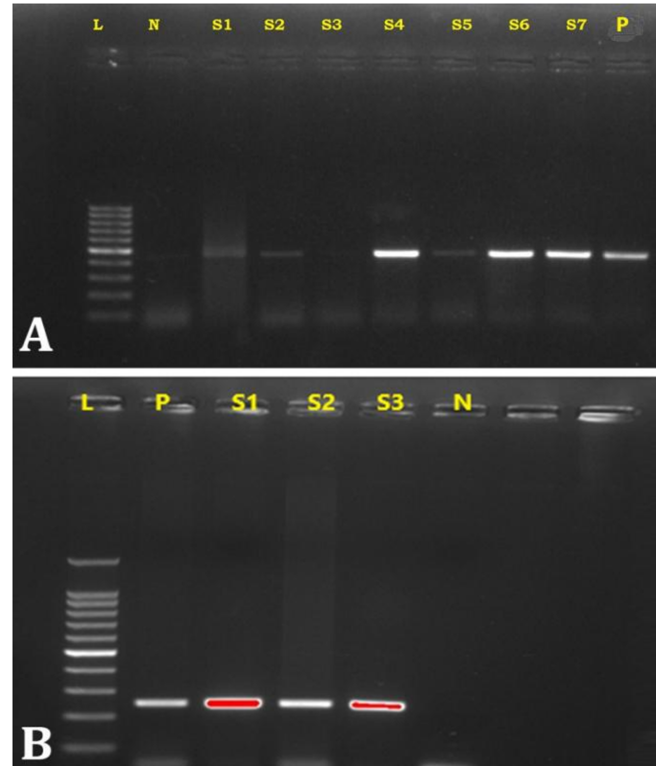

Fig. 5. Molecular confirmation of *Mycobacterium tuberculosis* complex (MTBC). Amplification of **A)** *Mycobacterium* genus-specific 441bp of *hsp*65 gene, and **B)** MTBC-specific 248 bp of *IS1081* insertion sequence of positive tissue samples in 1.50% agarose gel. **A:** L: Ladder (Thermo Scientific GeneRular 100 bp DNA Ladder); N: Non-template control; S1-S7: Samples; P: Positive control; **B:** L: Ladder (Thermo Scientific GeneRular 100bp DNA Ladder); P: Positive control (*Mycobacterium bovis* AN5); S1-S3: Samples; N: Negative control.

Discussion

Mycobacterium bovis is supposed to be the major causative pathogen of tuberculosis in animals. Clinically, the diagnosis of tuberculosis in domestic, as well as wildlife species, is very complicated. Tuberculosis in wild animals is frequently diagnosed at necropsy after natural death, with no prior suspicion of tuberculosis. It has been determined that wild animals can get the infection by preys or scavenged carcasses and disseminate the infection to other animals. In wildlife, *M. bovis* infection is usually diagnosed based on post-mortem lesions, histopathology, and pathogen isolation from suspected tuberculosis nodules.[17] Due to the challenges in routine monitoring of wild animals, most cases are detected only during necropsy. Herbivorous wild animals may acquire tuberculosis infection either through close contact with infected individuals of the same species or from diseased livestock in proximity to wildlife habitats. Therefore, close surveillance of both wild animals and peripheral livestock is essential for controlling tuberculosis in such settings.

In the present study, the affected nilgai and sambar deer exhibited clinical symptoms, such as cough, dyspnea, and nasal discharge. During post-mortem examination, the most common gross lesions were observed in the lungs and liver. Yellowish-white granulomatous lesions in the sambar deer and nilgai varied in size, ranging from miliary to large, were similar to those reported in other *Bovidae* species. The lesions in the thoracic cavity and lymph nodes presented as either purulent or dry, depending on their location within the body.[18,19] Similar pneumonic lesions associated with *M. bovis* infection have also been documented in other wild animals.[20] In the current finding, the liver of the nilgai was enlarged, with nodule formation and caseous necrosis, whereas the liver of the sambar deer was also enlarged, hyperemic, and associated with dark-colored fluid accumulation in the pericardial sac. This might be due to the presence of mycobacteria in the pericardium, triggering a delayed-type hypersensitivity (type IV) immune inflammatory reaction, causing increased vascular permeability, leading to leakage of plasma proteins and immune cells into the pericardial space. Comparable findings have also been reported in nilgai, including the darkening of heart with accumulation of pericardial fluid.

At the microscopic level, our observations regarding different microscopic lesions in lungs of nilgai and sambar deer due to the *M. bovis* infection were similar, including granulomatous lesions with central caseation surrounded by epithelioid cells, lymphocytes, multi-nucleated giant cells, and fibrinous encapsulation. These findings are in accordance with some earlier studies where same lesions have been investigated in rhinoceros and dairy cattle. [21,22] Additionally, chronic microscopic lesions in the kidneys, liver, and lungs of nilgai succumbed to bovine tuberculosis have also been reported.[23] In the present study, amplification of *mycobacterium* genus-specific *hsp65* gene along MTBC-specific *IS1081* insertion sequence proved to be a very suitable choice for identification and detection of MTBC group. Detection and identification of mycobacterial DNA in tissue samples from the dead animals were verified through amplification of a 441 bp fragment of the *hsp65* gene and a 248 bp fragment of the MTBC-specific *IS1081* sequence, respectively. In the present study, *IS1081* PCR was found to be more sensitive for detection of MTBC members in tissues which might be due to the multi-copy nature of the targeted sequence in *M. bovis*. The *IS1081* has also been shown to be 100% sensitive in detecting *M. bovis* in tissue confirmed for tuberculosis and can detect *M. bovis* from milk samples.[15] Additionally, PCR targeting the *RvD1-Rv2031c* genomic region successfully amplified a 500 bp fragment, being specific to *M. bovis* in MTBC-positive tissue samples. Similar analyses have been conducted to distinguish *M. bovis* from other MTBC members using the same 500 bp DNA fragment within the *RvD1-Rv2031c* genomic sequence.[24] These primers have been proven to be highly effective in detecting *M. bovis* DNA from blood samples with 100% accuracy compared to the traditional microbiological methods.[25,26] There is a growing concern regarding the misidentification of *M. bovis*, as many cases initially suspected to be *M. bovis* have been confirmed as *M. orygis*. Whole-genome sequencing provides a definitive means of differentiating between *M. bovis* and *M. orygis.*[27] Therefore, based on PCR detection of *hsp65* and *IS1081*, identification can only be reliably confirmed up to the MTBC level. Molecular approaches have been beneficial and the most promising alternative method for tuberculosis detection with regard to specificity and sensitivity.[28]

To the best of our knowledge, the pathological and molecular detection of tuberculosis in deer and nilgai in the present study represents the first documented case of the disease in these wildlife species from the Northeastern region of India. This finding highlights the urgent need for systematic surveillance and testing of other herd-forming wildlife species, as tuberculosis spreads easily among immuno-compromised populations living in close contact. Also, assessing the tuberculosis status of zookeepers and nearby livestock is crucial to identify potential sources of infection and implement measures to prevent its spread among wildlife. A comprehensive understanding of tuberculosis epidemiology requires an integrated approach combining clinical, pathological, microbiological, and molecular investigations, which can aid in achieving a definitive diagnosis of this challenging pathogen.

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Conflict of interest

This is to declare no conflict of interest.

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