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Original Article

Molecular identification and infection pathology of *Mycobacterium* spp. in captive wild animals in Pakistan

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Abstract

Introduction: Animal tuberculosis is an infectious, chronic, granulomatous, and debilitating disease affecting animals as well as humans. However, in recent decades, there have been many endemic geographic localities where animal tuberculosis has been identified in wildlife reservoirs, limiting the eradication program in cattle. This study aimed to identify animal tuberculosis in captive zoo animals in Pakistan.

Methodology: In total, 185 morbid zoo animals were brought for postmortem examination at a veterinary postmortem facility. During the macroscopic examination, these animals were thoroughly examined for the presence of suggestive gross lesions of animal tuberculosis (granulomas/tubercles), and the pattern and distribution of these lesions in different organs. The Ziehl-Neelsen (ZN) staining was performed on smears prepared from granulomatous lesions of lung tissue followed by molecular identification of *M. bovis* and *M. tuberculosis* DNA using polymerase chain reaction (PCR).

Results: The postmortem examination revealed that 8.1% (15/185) of animals had gross tuberculosis lesions on the lungs and lymph nodes. The ZN staining of tissue smears showed 5.40% positivity while *M. bovis* and *M. tuberculosis* DNA was identified in 3.78 % and 1.1% of investigated animals, respectively.

Conclusions: The study showed that animal tuberculosis is prevalent among wildlife in Pakistan and it may pose serious public health concerns to the people visiting these zoos and wildlife parks.

Key words: Tuberculosis; *M. bovis*; *M. tuberculosis*; wildlife; pathology; PCR.

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Introduction

Animal tuberculosis (TB) is a bacterial zoonosis of public health importance affecting wildlife, livestock, and humans throroughout the world. It is a chronic granulomatous disease caused by infectious organisms of the *Mycobacterium* (*M.*) *tuberculosis* complex, particularly, *M. bovis* and *M. tuberculosis* [1,2]. Clinically, TB is characterized by general weakness and weight loss, fever, cough, diarrhea, and enlargement of lymph nodes. Gross lesions in TB present as yellowish, cheesy, necrotic areas in the form of nodules of light grey to white fibrous tissues. Lesions may be purulent or dry with caseation or fibrosis and may be calcified or caseo-calcified [3]. The main sites of lesions vary between species but lungs with their related lymph nodes (LN) are common sites of lesions [4]. Histologically the tubercle has a central portion of caseous necrosis along with calcification encompassed by epithelioid cells, lymphocytes, plasma cells, and Langhans giant cells. There may be scattered foci of neutrophils and degenerated leucocytes on the junction between the surrounding mantle of inflammatory cells and caseo- necrotic centers [5].

Animal TB is prevalent in different wildlife species (wild boar, deer, elephants, African buffalo, African lions, mongoose, and wood bison) in various parts of the world including Africa, Europe, Asia, Australia, and North America [2,6]. Furthermore, animal TB has been reported in captive wild animals in various zoos and wildlife parks in different geographical regions. Tuberculosis caused by *M. tuberculosis* was reported in Asian elephants, giraffes, and Malayan tapir kept in captivity at different zoos and wildlife parks in Sweden, Thailand, and the USA, respectively [7-9]. In Pakistan, *M. bovis* and *M. tuberculosis* were identified as causing infections in antelopes at the Lahore Zoo [10]. Another study conducted on Brazilian captive bison revealed the tuberculosis infection caused by *M. bovis* [11]. Animal TB has been reported in captive and wild deer from the USA, Canada, New Zealand, England, Hungary, Denmark, Spain, France, Taiwan, Bangladesh, and India [12-14].

Animal TB is endemic and widely spreading in regions where livestock is grown extensively and is connected to commercialized activity particularly animal exchange [15]. Most animals like deer, badgers, ferrets, and foxes are considered reservoirs for transmission to livestock and humans [16]. In the USA, infected cattle are believed to play an important role in disease transmission while in New Zealand possums have remained a major source of infection [17]. Among animals, prevalence varies from as low as 5% to as high as 95% in various parts of the world [6,17]. In Pakistan, TB has been detected in wildlife in Islamabad Zoo and the results showed a prevalence of 3.6% and 3.2% in the Bovidae and Cervidae families, respectively [18]. Similarly, in Lahore Zoo, 30% and 20% of antelopes were found positive for tuberculosis caused by *M. bovis* and *M. tuberculosis*, respectively [10]. The epidemiology of tuberculosis is complex and may comprise transmission among various species of livestock, wildlife, and humans. The presence of infected wildlife, carrier animals, and geography are important risk factors for the occurrence and spread of tuberculosis among domestic animals, humans and wildlife [15,19,20,]. Inhalation and ingestion of contaminated raw dairy products remained major routes of infection in humans [21].

Diagnosis of animal TB is challenging and may include several diagnostic assays like in vivo delayed-type hypersensitivity the classical tuberculin skin test (TST), IFN gamma assay, bacteriological isolation, and molecular identification of bacterial DNA using polymerase chain reaction (PCR) [22]. Clinical diagnosis and use of routine serological tests like TST are not practicable in wildlife as handling and capturing of these animals is difficult and may put the animal under stress leading to serious consequences. The postmortem and histopathological diagnosis along with bacteriological and molecular identification under strict biosafety conditions is promising to diagnose animal TB. However, bacterial isolation is laborious, time-consuming, and also less sensitive. The identification of *Mycobacterium* DNA using PCR is a test of choice, easy to apply, and especially useful for the direct detection of mycobacterial species.

In Pakistan, the scientific data regarding postmortem and histopathological examination, and molecular identification of animal TB in captive wildlife and zoo animals are scarce. The current study aimed to investigate the animals infected with tuberculosis using gross and histopathological changes associated with tuberculosis and molecular identification of *M. bovis* and *M. tuberculosis* in zoo animals brought for necropsy examination.

Table 1. The details of various captive wildlife species brought for necropsy examination and included in the study.

Species	Public wildlife parks	Private wildlife parks
Blue bull	4	0
Hog deer	26	8
Spotted deer	20	6
Fallow deer	16	4
Fawn deer	3	0
Chinkara	35	10
Black buck	7	5
Gorilla	1	0
Guinea pig	2	0
Jackal	3	0
Wallaby	3	0
Monkey	5	0
Mouflon sheep	16	0
Lion	10	1

Methodology

Sample collection

The study was conducted at the postmortem block of the Pathology Department, University of Veterinary and Animal Sciences, Lahore, and the Department of Pathology, University College of Veterinary and Animal Sciences, Islamia University Bahawalpur. A total of 185 dead animals (March 2019 to March 2020) were brought for postmortem examination from various wildlife breeding parks, government and private zoos, and captive wild animals were included in the study (Table 1). Out of these, 15 animals showed lesions of suspected tuberculosis. The lung tissues of these suspected animals were further processed for histopathological and molecular identification.

Histopathology

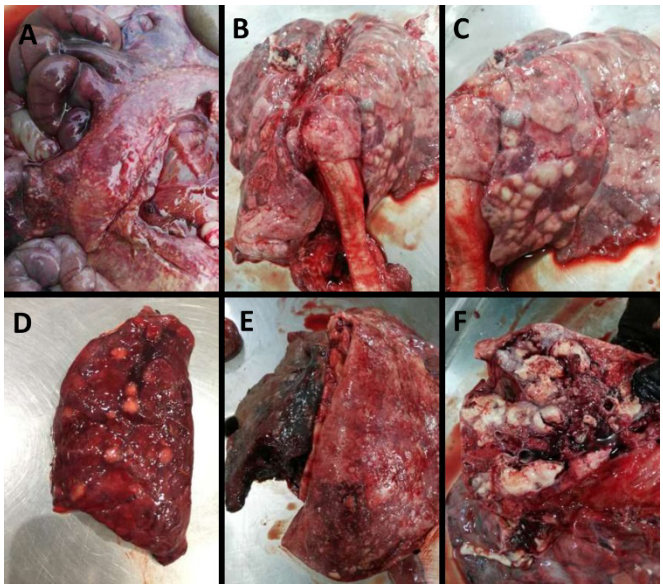
For every animal under investigation, 1 to 3 pieces of tissue (measuring 2 x 2 cm), were preserved in buffered formalin and fixed tissue sections were dehydrated using ascending grades of ethanol and embedded in paraffin. Approximately 5 µm thick sections were cut using a rotary microtome and stained by hematoxylin-eosin (HE) stains. The slides were observed under alight microscope for the presence of TB microscopic lesions [23].

Ziehl-Neelsen (ZN) staining and molecular identification

The smear was prepared from the lung tissue presenting granulomatous lesions and stained with ZN staining for the detection of microscopic presence of acid-fast bacilli of *Mycobacterium* [24].

Molecular identification of DNA of *Mycobacterium* species (*M. bovis* and *M. tuberculosis*) was performed using conventional PCR. The lung tissues were collected in 1.5 mL microfuge tubes and stored at -20 °C. The DNA was extracted using ready-to-use commercial kits according to the manufacturer's instructions (GeneJet Genomic DNA Purification Kit, Cat. No. K0721, Thermo Fisher Scientific, Waltham, USA). The PCR was performed with little modification of previously described protocols for the identification of *M. bovis* and *M. tuberculosis* DNA [18,25]. Separate PCR reactions were carried out using primer sets (JB21(TCGTCCGCTGATGCAAGTGC)-FW, JB22(CGTCCGCTGACCTCAAGAAG)-RV and pncATB-1.2 (ATGCGGGCGTTGATCATCGTC)-FW, pncAMT-2 (CGGTGTGCCGGAGAAGCGG)-RV for *M. bovis* (500 bp) and *M. tuberculosis* (185 bp), respectively. Briefly, a reaction mixture of 50 µL was prepared to contain 5 µL of *Taq* buffer, 300 mM dNTP, 1.5 mM $MgCl_2$, 2.5 U *Taq* polymerase (Thermo Fisher Scientific, Waltham, USA), 5% DMSO, 1.5 µL of each primer (20 µM) and 2 µL of template DNA [18]. Amplification of DNA was carried out by a gradient thermocycler (SCILOGEX SCI 1000-S, Rocky Hill, USA). The reaction conditions were initial denaturation at 94 °C for 4 min followed by 35 cycles of denaturing at 94 °C for 30 s, annealing at 55 °C for 1 min and extension at 73 °C for 1 min and final extension at 72 °C for 10 min. The PCR products were analyzed on 1% agarose gel, stained with ethidium bromide, and visualized under UV light (Alpha Imager, Alpha Innotech Corporation, San Leandro, USA). Distilled water was used as the negative control and *M. bovis* and *M. tuberculosis* DNA as the positive control.

Figure 1. A: A mesenteric lymph node showing small raised millet like nodules; **B** and **C**: Lung tissue from a deer showing raised off-white nodules on the anterior area; **D:** Lungs from a deer showing tuberculoid lesions disseminated on the entire surface of lungs at variable distances; **E:** Lungs from a zoo animal showing consolidation, red hepatization, and granulomas of varying size; **F:** Cut surface of a lung tissue showing cream colored exudate in the lung parenchyma.



Results

Macroscopic/gross examination

Out of 185 animals received for postmortem examination, 15 animals including hog deer (n = 2), spotted deer (n = 3), fallow deer (n = 3), chinkara (n = 5), and black buck (n = 2) were suspected of tuberculosis based on gross lesions of bovine TB. Physical examination and clinical history suggested that these animals were emaciated, gradually lost body condition, lethargic, weak, and were showing signs of respiratory distress. Gross examination showed the presence of circumscribed yellowish-white nodules varying in numbers and size mainly in the lungs and associated lymph nodes (Figure 1). The cut sections of the nodules showed thick, creamy to yellowish inspissated necrotic material with a gritty sensation on cutting. In one animal (fallow deer), small miliary tubercles were noticed on the mesenteric lymph nodes without any lesions in other organs.

Histopathological/microscopic examination

Microscopic examination of HE-stained tissue sections revealed lesions suggestive of bovine tuberculosis. These lesions consisted of large central areas of caseous necrosis that were surrounded by a narrow to moderately thickened layer of mixed inflammatory cells and fibrous connective tissue

proliferation in some cases. Inflammatory cells consisted of focal aggregation of macrophages, lymphocytes, and plasma cells, and small numbers of giant cells were seen in a few animals (Figure 2). Mineralization was present at the center of the necrotic foci in most of the cases while in a few, peripheral mineralization of the necrotic foci was also observed which was extended to the band of inflammatory cells. Many lesions had a large number of neutrophils and abundant chromatin debris scattered in the necrotic material and concentrated at the periphery of the necrotic area.

ZN staining and molecular confirmation

ZN staining revealed acid-fast bacilli in 66.6% (10/15) of impression smears prepared from lesions of lungs and lymph nodes suggestive of animal TB (Figure 2A inset).

Mycobacterial DNA was identified in 9 samples including *M. bovis* (7 samples) and *M. tuberculosis* (2 samples) using species-specific PCR. The presence of 500 bp (*M. bovis*) and 185 bp (*M. tuberculosis*) amplicon size was observed on the agarose gel (Figure 3). Overall, 8.1%, 5.4%, and 4.86% of specimens were found positive by gross examination and histopathological examination, ZN staining, and PCR, respectively (Table 2).

Figure 3. Image of PCR amplified products visualized on agarose gel. M = 100 bp ladder; *M. bovis* control (500 bp), *M. tuberculosis* (M. tb) control (185 bp); S1: Sample positive for *M. bovis* and S2, S3: Sample positive for **M. tuberculosis**.

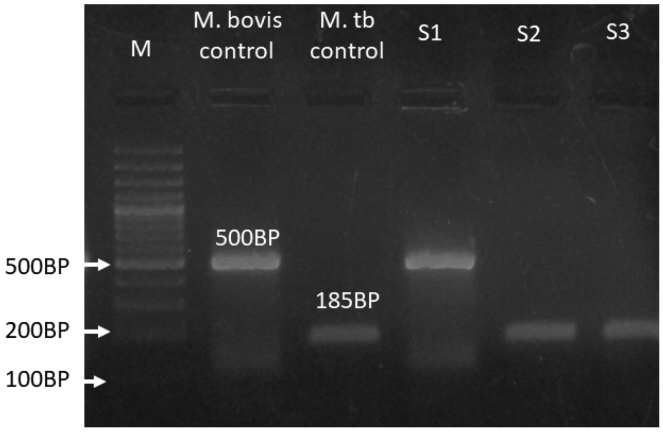


Figure 2. A: A low magnification image from the lung tissue of an animal suffering from tuberculosis showing a typical granuloma with central caseous necrotic area and surrounded by mononuclear inflammatory cells (Objective 4x). Inset: Acid fast staining of smear prepared from caseous tuberculous lesions showing acid fast bacilli (Ziehl-Neelsen Stain, Objective 100x); **B:** Microphotograph from lung tissue affected with tuberculosis showing a caseo-necrotic area surrounded by mononuclear inflammatory cells. Inset: Many mononuclear cells including lymphocytes, macrophages, and giant cell are visible (Objective 40x)

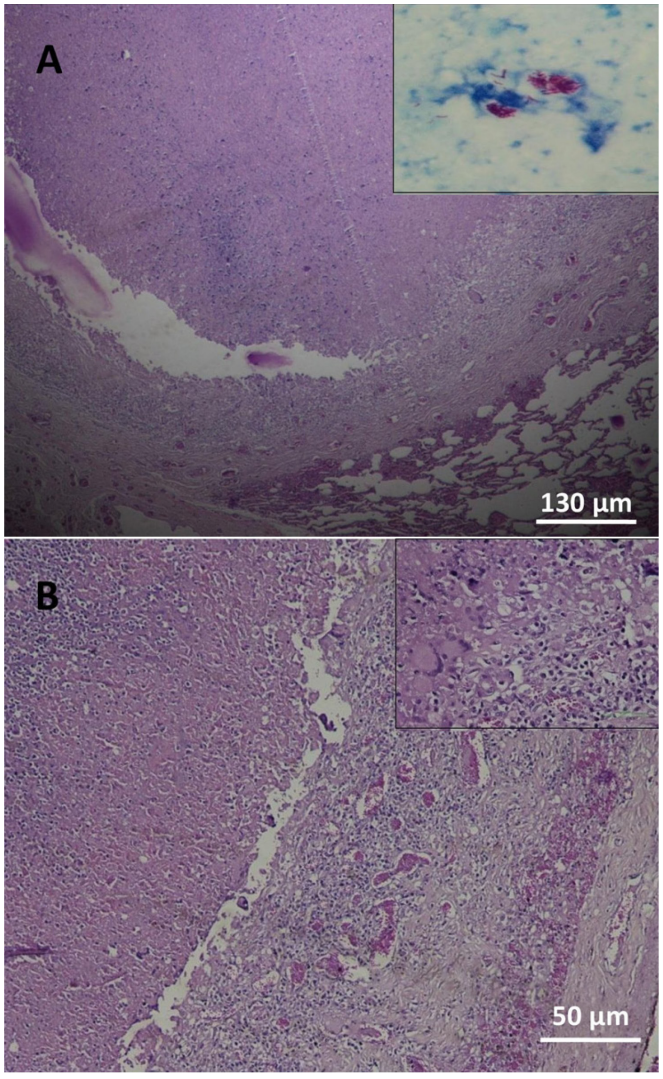


Table 2. Statistical analysis of the data obtained through analysis of gross lesions, ZN staining and PCR.

Animal locality	Animal tested (N)	Positivity based on lesions				Positivity based on ZN*				Positivity based on PCR[a]				Positivity based on PCR[b]			
		n (+)	%*	CI*	Sig.	n (+)	%	CI	Sig.	n (+)	%	CI	Sig.	n (+)	%	CI	Sig.
Government zoo	151	10	6.62	± 0.04	0.12	7	4.64	± 0.03	0.33	4	2.64	± 0.03	0.08	2	1.32	± 0.08	0.5
Private zoo	34	5	14.7	± 0.12		3	8.82	± 0.09		3	8.82	± 0.09		0	0	± 0.0	
Total	185	15	8.10	± 0.04		10	5.51	± 0.03		7	3.78	± 0.14		2	1.08	± 0.02	

%: Percentage prevalence; CI: Confidence interval (95%); ZN: Ziehl-Neelsen positive; PCR[a,b:] Polymerase chain reaction, where a: *M. bovis* and b: *M. tuberculosiss.*

Discussion

This study is the first investigation of animal TB in captive wildlife in Pakistan using molecular methods in addition to gross and histopathological examination. Animal TB is caused by various *Mycobacterium* species particularly *M. bovis* and *M. tuberculosis* which have a wide host range including domestic (cattle) and wildlife (deer, chinkara, wild buck, etc.) in various parts of the world across different ecosystems. The disease is rapidly spreading owing to poor management, overcrowding, and animal movement in developing countries [26]. Although, this disease has been effectively controlled in domestic animals (cattle) in developed countries, infection of wildlife, especially deer, remains a serious concern for the tuberculosis eradication and control programs in many parts of the world particularly in developing countries like Pakistan. The disease is categorized as a neglected zoonotic disease by the World Health Organization (WHO) which causes serious health problems in animals and is highly infectious to humans. Humans can contract the infection by direct contact with animals or by consuming contaminated raw animal products [27].

Diagnosis of TB in animals, particularly in free-range wild animals or captive wildlife, is necessary for the effective control and public health management of the disease. Multiple factors including capturing or handling wild animals for sample collection and diagnosis are a big challenge for field veterinarians and public health officials. Microbial culture, serological assays like TST, gross and microscopic examination of lesions, ZN staining, and PCR are considered important tools for diagnosis and surveillance of wild animals [2].

In the current research, 185 animals were examined by gross and histopathology which was further confirmed by ZN staining and molecular identification of *M. bovis* and *M. tuberculosis* DNA by PCR. Around 8.1% of animals showed animal TB lesions in postmortem examination and were confirmed by histopathology. Furthermore, 5.4% of specimens that were examined using ZN staining showed acid-fast bacilli. Molecular assays confirmed the presence of *M. bovis* (3.78%) and *M. tuberculosis* (1.1%) DNA in 4.86% of total samples.

The gross lesions of animal TB in the present study were characterized as yellowish, cheesy, necrotic areas in the form of nodules of light grey to white color and were mainly restricted to lungs and associated lymphoid tissue. Though lesions of TB may be present in other organ systems too, thoracic cavity is the usual site of infection in most animals [3]. Histological examination of the suspected cases showed necrotic granulomas with infiltration of inflammatory cells which were mainly mononuclear but, in some cases, admixed with neutrophils. The multinucleated giant cells were not numerous as observed in a previous study on British deer [28]. As all the suspected cases were not confirmed with ZN staining and PCR, diagnosis of TB shall not be made based on pathological analysis of the tissues only [22]. The other causes of the caseation in the lungs e.g., *Fusobacterium necrophorum* and *Corynebacterium pseudotuberculosis* shall also be considered [29]. In the current study, a few of the cases negative for tuberculosis on PCR were found positive for caseous lymphadenitis.

Out of 15 specimens collected from the TB suspected animals, 10 were found positive using ZN staining. The findings in the current study are in agreement with the previous studies [19,30]. ZN staining is less time-consuming; however, it provides lower specificity as compared to molecular tools like PCR [31]. The overall result of the current study showed 4.86% (9/185) of the animals under study were positive for *M. bovis* and *M. tuberculosis* using PCR. In a study conducted at Lahore Zoo and wildlife parks located in Lahore, a high incidence of *M. bovis* (30%) and *M. tuberculosis* (20%) was observed among antelopes [10]. The findings of the current study showing the involvement of *M. bovis* and *M. tuberculosis* in animal TB are in agreement with previous findings showing a higher incidence of *M. bovis* in Pakistan as compared to *M. tuberculosis*. This study further demonstrated that *M. bovis* is more prevalent in Pakistan as compared to *M. tuberculosis* in animals kept in captivity. These studies also suggest that captive wild animals may be an important source of infections by *M. bovis* and *M. tuberculosis* which is a concern for public health.

Although, the sensitivity and specificity of TST are lower than the molecular assays, a study conducted at Islamabad Zoo using TST showed 3.3% positivity which is in agreement with the current study [32]. This variation in the incidence of the disease may be attributable to geography, use of diagnostic tools, and study design as the current study was focused on the animals brought for postmortem examination only.

Tuberculosis in animals has a dual impact as it not only affects the health and productivity of animals but may also be transmitted to humans. In Pakistan, *M. bovis* has also been detected in abattoir workers and livestock farmers [33]. Tuberculosis in zoo animals has been reported in many parts of the world and is considered a reservoir of the disease for transmission to

livestock and humans in various parts of the world as well as in Pakistan [2,3].

Furthermore, it is assumed that the small sample size subjected to PCR may have resulted in low identification of *Mycobacterium* species in the current study in comparison to previous studies [10]. The authors believe that this may be the limitation of this study as the aim of the current study was only to observe tuberculosis in captive animals brought for postmortem rather than epidemiological or surveillance studies. The presence of *Mycobacterium* species (*M. bovis* and *M. tuberculosis*) in wild and zoo animals in Pakistan is alarming and may pose serious impacts on public health. Further epidemiological studies are needed to investigate the true prevalence of tuberculosis and molecular identification of other *Mycobacterium* species in wild and captive animals. Additionally, investigations are also needed to establish whether this disease is endemic in wildlife or the source of infection is humans or domestic animals and vice versa [34-36].

Conclusions

To the best of our knowledge, this is the first study reporting the presence of *M. bovis* and *M. tuberculosis* DNA in captive wild animals along with postmortem and histopathological investigations. The study showed the prevalence of animal TB along with the distribution of lesions in the lungs and lymph nodes. The current study also provides insight into the occurrence of TB in various wild animals and the potential risk it poses to human health. In Pakistan, the trend of wildlife parks and zoos has increased in the recent past due to urbanization and the movement of a large number of people to urban areas. The increasing importance of wildlife also poses risk to the community and hence regular epidemiological, microbiological and pathological studies are needed to monitor disease trends like tuberculosis in these animals. Zoonotic diseases like animal tuberculosis require continuous monitoring and research on pathogen transmission and pathogenesis of various *Mycobacterium* species including *M. bovis* and *M. tuberculosis* infection in the various captive wildlife species.

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References

1. Sima BT, Belachew T, Abebe F (2017) Knowledge, attitude and perceived stigma towards tuberculosis among pastoralists. Do they differ from sedentary communities? A comparative cross-sectional study. PloS One 7: e0181032. doi: 10.1371/journal.pone.0181032.
2. Thomas J, Balseiro A, Gortázar C, Risalde MA (2021) Diagnosis of tuberculosis in wildlife: a systematic review. Vet Res 1: 1-23. doi: 10.1186/s13567-020-00881-y.
3. Domingo M, Vidal E, Marco A (2014) Pathology of bovine tuberculosis. Res Vet Sci 97: S20-S29. doi: 10.1016/j.rvsc.2014.03.017.
4. O'Brien DJ, Fitzgerald SD, Lyon TJ, Butler KL, Fierke JS, Clarke KR, Schmitt SM, Cooley TM, Berry DE (2001) Tuberculous lesions in free-ranging white-tailed deer in Michigan. J Wildl Dis 3: 608-613. doi: 10.7589/0090-3558-37.3.608.
5. Lambert S, Hars J, Réveillaud E, Moyen J-L, Gares H, Rambaud T, Gueneau E, Faure E, Boschiroli M-L, Richomme C (2017) Host status of wild roe deer in bovine tuberculosis endemic areas. Eur J Wildl Res 1: 1-9. doi: 10.1007/s10344-016-1071-4.
6. Fitzgerald S, Kaneene J (2013) Wildlife reservoirs of bovine tuberculosis worldwide: hosts, pathology, surveillance, and control. Vet Pathol 3: 488-499. doi: 10.1177/0300985812467472.
7. Kaewamatawong T, Banlunara W, Rungsipipat A, Pirarat N, Puranaveja S, Sommanustweechai A (2010) Disseminated tuberculosis in captive Malayan tapir (*Tapirus indicus*). Thai J Vet Med 4: 427-431.
8. Mikota SK, Peddie L, Peddie J, Isaza R, Dunker F, West G, Lindsay W, Larsen RS (2001) Epidemiology and diagnosis of *Mycobacterium tuberculosis* in captive Asian elephants (*Elephas maximus*). JZWM 1: 1-16.
9. Sternberg Lewerin S, Eld K, Bölske G, Olsson S L, Röken B, Ghebremichael S, Koivula T, Källenius G (2005) Outbreak of *Mycobacterium tuberculosis* infection among captive Asian elephants in a Swedish zoo. Vet Record 6: 171-175. doi: 10.1136/vr.156.6.171.
10. Akhtar R, Sadiqa M, Tipu MY, Khan MR, Aslam A, Ijaz M, Mustafa G, Zahid B (2019) Use of molecular probes for presumptive diagnosis of tuberculosis associated with *Mycobacterium tuberculosis* and *Mycobacterium bovis* infection in antelopes in Pakistan. Pak Vet J 52: 470. doi: 10.29261/pakvetj/2019.067.
11. Zimpel CK, Brum JS, de Souza Filho AF, Biondo AW, Perotta JH, Dib CC, Bonat M, Neto JSF, Brandão PE, Heinemann MB (2017) *Mycobacterium bovis* in a European bison (*Bison bonasus*) raises concerns about tuberculosis in Brazilian captive wildlife populations: a case report. BMC Res Notes 1: 1-6. doi: 10.1186/s13104-017-2413-3.
12. Hota SR, Sahu SK, Behera BK, Pahari A, Sahoo N (2020) Microbiological and molecular diagnosis of *Mycobacterium tuberculosis* infection in spotted deer (*Axis axis*) of an Indian zoo. Int J Curr Microbiol App Sci 6: 844-851. doi: 10.20546/ijcmas.2020.906.107.
13. Palmer MV, O'Brien DJ, Griffin JF, Nugent G, Lisle GD, Ward A, Delahay RJ (2015). Tuberculosis in wild and captive deer. In Mukundan H, Chambers MA, Waters WR, Larsen MH, editors. Tuberculosis, leprosy and mycobacterial diseases of man and animals: the many hosts of mycobacteria. Wallingford UK: CABI. 334-364. doi: 10.1079/9781780643960.0334.

14. Sultana S, Sultana N, Islam M, Pervin M, Khan MAI, Khan MAHNA (2021) Zoonotic diseases appeared to be a major hurdle to successful deer farming in Bangladesh. Vet World 9: 2462. doi: 10.14202/vetworld.2021.2462-2472.
15. Telles EO, Neto JSF, Galvis JOA, Grisi-Filho JHH, da Costa D, Said ALPR, Amaku M, Dias RA, Ferreira F, Gonçalves VSP (2016) Epidemiologic characterization of bovine tuberculosis in the state of Espírito Santo, Brazil. Semina: Ciências Agrárias 5: 3567-3578. doi: 10.5433/1679-0359.2016v37n5Supl2p3567.
16. Yasmin A, Hossain M, Rima U, Ruba T, Khan M (2017) Detection of specific causes of tuberculosis in the dairy cattle of Bangladesh Agricultural University by sequencing and sequence analysis. BJVM 1: 13-20. doi: 10.3329/bjvm.v15i1.34049.
17. Nugent G, Gortázar C, Knowles G (2015) The epidemiology of *Mycobacterium bovis* in wild deer and feral pigs and their roles in the establishment and spread of bovine tuberculosis in New Zealand wildlife. N Z Vet J 63 Suppl 1: 54-67. doi: 10.1080/00480169.2014.963792.
18. Nawaz A, Chaudhry Z, Shahid M, Gul S, Khan F, Hussain M (2012) Detection of *Mycobacterium tuberculosis* and *Mycobacterium bovis* in sputum and blood samples of human. J Agr Sci 22: 117-120.
19. Carstensen M, DonCarlos MW (2011) Preventing the establishment of a wildlife disease reservoir: a case study of bovine tuberculosis in wild deer in Minnesota, USA. Vet Med Int 2011: 1-10. doi: 10.4061/2011/413240.
20. Torres-Gonzalez P, Cervera-Hernandez M E, Martinez-Gamboa A, Garcia-Garcia L, Cruz-Hervert L P, Bobadilla-del Valle M, Ponce-de Leon A, Sifuentes-Osornio J (2016) Human tuberculosis caused by *Mycobacterium bovis*: a retrospective comparison with *Mycobacterium tuberculosis* in a Mexican tertiary care centre, 2000-2015. BMC Infect Dis 1: 1-9. doi: 10.1186/s12879-016-2001-5.
21. Barasona JA, Vicente J, Díez-Delgado I, Aznar J, Gortázar C, Torres MJ (2017) Environmental presence of *Mycobacterium tuberculosis* complex in aggregation points at the wildlife/livestock interface. Transbound Emerg Dis 4: 1148-1158. doi: 10.1111/tbed.12480.
22. Ramos D, Silva P, Dellagostin O (2015) Diagnosis of bovine tuberculosis: review of main techniques. Bra J Biol 75: 830-837. doi: 10.1590/1519-6984.23613.
23. Martín-Hernando M P, Höfle U, Vicente J, Ruiz-Fons F, Vidal D, Barral M, Garrido JM, de la Fuente J, Gortazar C (2007) Lesions associated with *Mycobacterium tuberculosis* complex infection in the European wild boar. Tuberculosis 4: 360-367. doi: 10.1016/j.tube.2007.02.003.
24. Saidu AS, Okolocha E, Gamawa A, Babashani M, Bakari N (2015) Occurrence and distribution of bovine tuberculosis (*Mycobacterium bovis*) in slaughtered cattle in the abattoirs of Bauchi State, Nigeria. Vet World 3: 432. doi: 10.14202/vetworld.2015.432-437.
25. Huard RC, de Oliveira Lazzarini LC, Butler WR, van Soolingen D, Ho JL (2003) PCR-based method to differentiate the subspecies of the *Mycobacterium tuberculosis* complex on the basis of genomic deletions. J Clini Microbiol 4: 1637-1650. doi: 10.1128/JCM.41.4.1637-1650.2003.
26. Brook RK, McLachlan SM (2006) Factors influencing farmers' concerns regarding bovine tuberculosis in wildlife and livestock around Riding Mountain National Park. J Environ Manage 2: 156-166. doi: 10.1016/j.jenvman.2005.08.022.
27. LoBue P, Enarson D, Thoen C (2010) Tuberculosis in humans and animals: an overview. Serialized article. Tuberculosis: a re-emerging disease in animals and humans. Number 1 in the series. Int J Tuberc Lung Dis 14: 1075-1078.
28. Johnson L, Liebana E, Nunez A, Spencer Y, Clifton-Hadley R, Jahans K, Ward A, Barlow A, Delahay R (2008) Histological observations of bovine tuberculosis in lung and lymph node tissues from British deer. Vet J 3: 409-412. doi: 10.1016/j.tvjl.2007.04.021.
29. Caswell JL, Williams KJ (2016) Respiratory System, In Maxie MG editor. Jubb, Kennedy and Palmer's Pathology of Domestic Animals: Philadelphia. Saunders. 2: 465-591. doi: 10.1016/B978-0-7020-5318-4.00011-5.
30. Schmitt SM, Fitzgerald SD, Cooley TM, Bruning-Fann CS, Sullivan L, Berry D, Carlson T, Minnis RB, Payeur JB, Sikarskie J (1997) Bovine tuberculosis in free-ranging white-tailed deer from Michigan. J Wildl Dis 4: 749-758. doi: 10.7589/0090-3558-33.4.749.
31. Cho M S, Lee S N, Sung S H, Han W S (2003) Comparison of Ziehl-Neelsen Stain stain and TB-PCR on detection of *Mycobacterium tuberculosis* in formalin-fixed, paraffin-embedded tissues of chronic granulomatous inflammation. Korean J Pathol 6: 379-383.
32. Shahid A, Tariq Javed M, Nisar Khan M, Cagiola M (2012) Prevalence of bovine tuberculosis in zoo animals in Pakistan. Iran J Vet Res 1: 58-63.
33. Khattak I, Mushtaq M, Ahmad M, Khan M, Haider J (2016) Zoonotic tuberculosis in occupationally exposed groups in Pakistan. Occup Med (Oxford, England) 5: 371-376. doi: 10.1093/occmed/kqw039.
34. Aranaz A, De Juan L, Montero N, Sánchez C, Galka M, Delso C, Alvarez J, Romero B, Bezos J, Vela AI (2004) Bovine tuberculosis (*Mycobacterium bovis*) in wildlife in Spain. J Clin Microbiol 6: 2602-2608. doi: 10.1128/JCM.42.6.2602-2608.2004.
35. Buddle B, Wedlock D, Denis M (2006) Progress in the development of tuberculosis vaccines for cattle and wildlife. Vet Microbiol 2-4: 191-200. doi: 10.1016/j.vetmic.2005.11.027.
36. Buddle BM, Wedlock DN, Denis M, Vordermeier HM, Hewinson RG (2011) Update on vaccination of cattle and wildlife populations against tuberculosis. Vet Microbiol 1-2: 14-22. doi: 10.1016/j.vetmic.2011.02.021.

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