Mycobacterial infection in a Nile crocodile (*Crocodylus niloticus*) from Türkiye

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ABSTRACT

Mycobacterial infection in Nile crocodile tissues sent from a private zoo was characterized pathomorphologically and immunohistochemically in this case. Macroscopically, multifocal, greyish-white areas ranging in size from 1 mm to 5 mm were seen in the lung, liver, and spleen. Histologically, a large number of well-demarcated necrotic areas were seen. These areas included nuclei debris locally. Inflammatory cells along with a couple of multinucleated giant cells surrounded the necrotic cores. Numerous acid-fast bacilli were detected by Ziehl-Neelsen staining method. Immunolabelling for both *Mycobacterium bovis* and anti-BCG antibodies was positive in each tissue.

Mycobacterial infection is an important disease caused by *Mycobacterium* spp. in a wide range of species (5). There are more than 140 species of non-tuberculous mycobacteria in humans and animals (8). *Mycobacterium* spp. are the most common aetiological agent for necrotising granulomatous inflammation (11). It has been reported that mycobacterial infections are commonly seen in reptiles; however, this infection is extremely rare in crocodiles (5). Heterophilic, histiocytic and chronic granulomas associated with *Mycobacterium* were described in reptiles such as snake, chelonian and lizard (13). The most isolated mycobacterial pathogens in crocodilians are summarised in Table 1. Mycobacterial infections in crocodiles have been reported in The United Kingdom (4), South Africa (6), Australia (1, 2), Netherlands (7, 16), South Korea (10) and Czech Republic (12). In crocodiles, the source of this infection is not known clearly. Presumably, its origin was fish for *M. fortuitium* and pork for *M. avium* (6). There is no known successful treatment of this disease (5). Although Ziehl-Neelsen staining is usually sufficient for the diagnosis of *Mycobacterium* spp., many studies show that additional tests such as Polymerase chain reaction (PCR) method are also needed (13, 14). It was realised that the immunohistochemistry (IHC) technique was not performed in any of the past studies concluded in crocodiles for *mycobacterium* diagnosis. The purpose of the case was to evaluate mycobacterial infection in a Nile crocodile with histopathological and immunohistochemical findings.

A private zoo provided lung, liver, and spleen samples in 10% neutral buffered formalin solution for pathological analysis. The samples were taken from a four-year-old female Nile crocodile (*Crocodylus niloticus*) with no prior clinical signs. After routine tissue processing, 5 µm sections were stained with standard Haematoxylin & Eosin (H&E) and Ziehl-Neelsen (ZN)

method for detection of acid-fast bacteria (9). The accurate diagnosis of mycobacterial infection was confirmed by Avidin-Biotin Complex Peroxidase (ABC-P; UltraVision Quanto Detection System HRP Polymer, Thermo Scientific, Catalog#TL-125-QHL) method. After deparaffinization and rehydration, the sections were incubated with 0.1% trypsin for 10 minutes at 37 °C. Endogenous peroxidase was blocked using 3% Hydrogen peroxide-methanol for 20 minutes at room temperature. Protein blocking solution was applied to sections for 10 minutes at 37 °C. Sections were then incubated with both *Mycobacterium bovis* (dilution ratio: 1:500, Dako) and anti-BCG (dilution ratio: 1:1000, Dako) antibodies in humidity chamber for 1 hour at 37 °C (15). Subsequently, sections were incubated with biotinylated antibody and streptavidin-peroxidase for 15 minutes at 37 °C. Sections were covered using 3-Amino-9-EthylCarbazole (AEC) chromogen for 7 minutes. Mayer's Haematoxylin was used as the counterstain for 3 minutes. For negative control slides, the primary antibody was substituted with both Phosphate-Buffered Saline (PBS) and mouse anti-rabbit IgG (dilution ratio: 1:100, Santa Cruz Biotechnology). Except for protein blocking, sections were washed with PBS between each step.

Gross examination revealed multifocal, greyish-white foci ranging from 1mm to 5 mm in diameter on the cut sections of all tissue samples (Figure 1). In H&E stained, multiple well-demarcated necrotic areas consisting of nuclei debris and peripherally infiltrated by a few amount of lymphocytes and macrophages were noticed in the lung, liver and spleen (Figure 2). Around necrotic areas, multinucleated giant cells were observed. Additionally, abundant intralesional acid-fast bacilli were demonstrated with Ziehl-Neelsen staining (Figure 3). Positive staining for *M. bovis* in immunohistochemistry is mostly found around necrotic areas and in macrophages. (Figure 4). Besides, positive immunoreaction for anti-BCG was also observed.

Typical granuloma structures have a central core of cell debris surrounded by multinucleated giant cells, macrophages and lymphocytes, which are limited to a fibrous connective tissue. They are defined in mycobacterial infections in crocodiles as in humans and other domestic animals; on the other hand, necrosis and low cellularity were striking histologic features in the case. Similar to other authors' findings, dystrophic calcification was also not noticed in this case (1, 2, 7, 10).

Table 1 Mycobacterial pathogens in crocodilians.

Species	Affected tissues	Inflammation type	Methods	Agent isolated	References
Caiman sclerops	Lung, spleen, liver, kidney, pancreas, testis, epiglottis	Granulomatous	Bacterial culture	*M. marinum*	(4)
Caiman sclerops	Kidney	Granulomatous	-	-	(16)
Crocodylus johnstoni	Lung*, liver, spleen*, kidney	Granulomatous	Gram, PAS, ZN and PCR*	*M. ulcerans*	(1)
Crocodylus porosus and *Crocodylus johnstoni*	Skin (snout, conjunctiva, jaws, neck, thigh)	Granulomatous	ZN	*Mycobacterium* spp.	(2)
Crocodylus niloticus	Liver, several organs Generalize Lungs Skin	Granulomatous Granulomatous Granulomatous Ulceration	Bacterial culture	*M. avium cplx* *M. terrae* *Atypical M.* *M. triviale*	(6)
Caiman	Fat	Granulomatous	Bacterial culture	*M. fortuitum*	(6)
Crocodylus johnstoni	Lung	Granulomatous	Fite's method, ZN and Nested PCR	*M. szulgai*	(10)
Caiman crocodilus fuscus	Liver, lung, spleen	Granulomatous	ZN and PRA	*M. szulgai* *M. chelonae*	(12)
Caiman latirostris	Intestinal wall, liver, spleen	Granulomatous	ZN and PCR	*M. intracellulare*	(7)
Crocodile	Lung, heart	No obvious lesions	PCR	*M. szulgai*	(3)

PAS, periodic acid-schiff; PCR, Polymerase Chain Reaction; PRA, PCR restriction analysis; ZN, Ziehl-Neelsen.

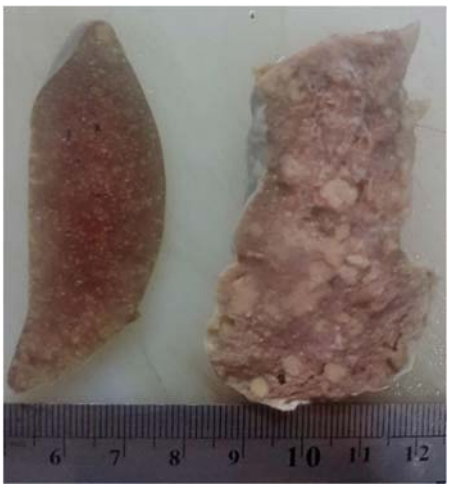

Figure 1. Multifocal, greyish-white areas in liver (left) and lung (right).

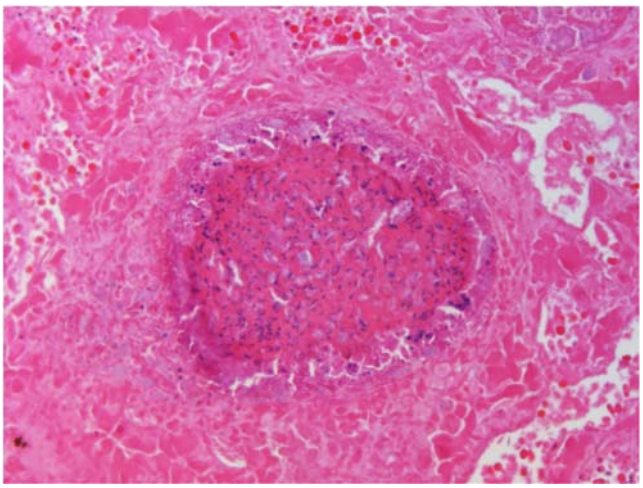

Figure 2. Well-demarcated necrotic area included nuclei debris and surrounded by lymphocytes, macrophages and multinucleated giant cell. Lung. H&E. X200.

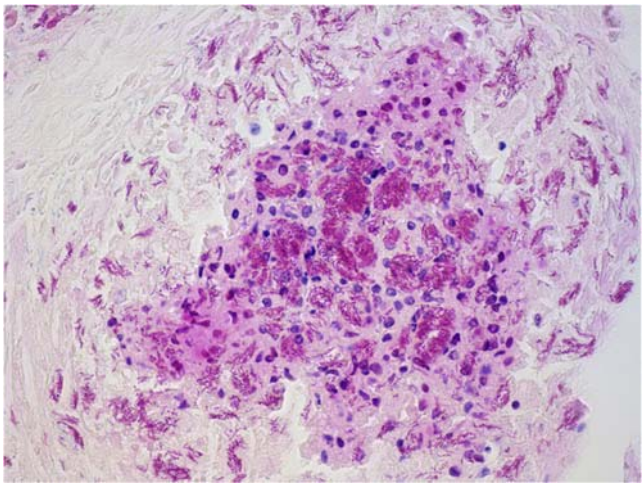

Figure 3. Numerous intralesional acid-fast bacilli. Liver. ZN. X400.

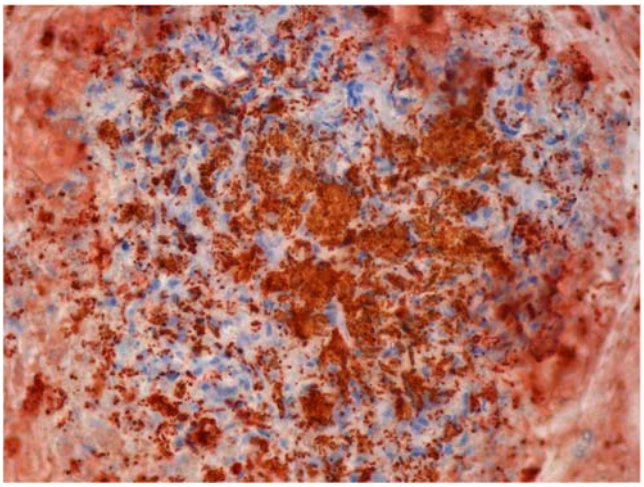

Figure 4. Prominent positive reaction for *M. bovis* both around the necrotic area and in macrophages. Lung. IHC. X400.

Generally, acid-fast bacteria have been found in the necrotic area and within the multinucleated giant cells (1, 2, 7, 10, 12). Roh et al. (2010) identified the microorganisms by Fite's method instead of Ziehl-Neelsen staining. However, in this study the bacteria were identified in the necrotic areas and in macrophages with Ziehl-Neelsen staining. In addition, other acid-fast bacteria, other than *Mycobacterium spp*., such as *Nocardia spp*., must always be remembered in Ziehl-Neelsen staining diagnosis. Reptiles including crocodiles are not vulnerable to *M. tuberculosis* and *M. bovis* infections due to their low body temperature (6). In this case we demonstrated the immunopositivity for *M. bovis*. Traditional bacterial culture and molecular techniques have been the gold standard for identifying the infectious agent. In the study, the tissues were sent within the formalin and we performed the immunohistochemistry method for demonstration of the causative agent. Because formalin-fixed tissues lack sensitivity and specificity, particularly for PCR, the study was based on immunohistochemistry to detect mycobacterial infection in crocodile, which resulted in the first diagnosis of mycobacterial infection in crocodile using the immunohistochemistry technique. Hereby, we also reported the first case of mycobacterial infection in a Nile crocodile in Türkiye.

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Ethical Statement

No ethical approval was required in this case report.

Conflict of Interest

The authors declared that there is no conflict of interest.

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