Mycobacterial infection in a Nile crocodile (Crocodylus niloticus) from Turkey

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Article History
Received: 02.07.2022
Accepted: 10.03.2023
DOI: 10.33988/avufd.1139830

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.

Keywords
Immunohistochemistry
Mycobacterial infection
Mycobacterium
Nile crocodile

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How to cite this article: Ahlat O, Özoner O, Filikci K, Atalay Vural S (XXXX): Mycobacterial infection in a Nile crocodile (Crocodylus niloticus) from Turkey. Ankara Univ Vet Fak Derg, XX (X), 000-000. DOI: 10.33988/avufd.1139830.

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 aetiologic 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. fortuitum 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)
cromogen for 7 minutes. Maye'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 1 mm 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.

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

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 seen 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 Turkey.

Acknowledgements
This study has been presented as an oral presentation at The 6th International Congress on Veterinary and Animal Sciences held online on 02-04 September 2021.

Financial Support
This research received no grant from any funding agency/sector.
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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