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Case report

Granulomatous pneumonia in a Nile crocodile (*Crocodylus niloticus*) caused by a member of *Mycobacterium chelonae/abscessus* group

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Abstract: A 40 years old male Nile crocodile (*Crocodylus niloticus*) was diagnosed with pulmonary mycobacteriosis caused by a member of *Mycobacterium chelonae/abscessus* group. Post-mortem examination showed a severe systemic visceral granulomatous involvement, with lesions in lungs, heart, liver, spleen and kidneys. Histopathological examination of lung, spleen, heart and liver revealed multifocal to coalescing granulomas showing eterophils in central zone and outer rim of epithelioid histiocytes, multinucleated giant cells and lymphocytes. The Ziehl–Neelsen histological staining revealed rare vacuoles containing numerous alcohol-acid resistant bacteria. Mycobacterial infection was confirmed by culture and PCR targeting rRNA 16S gene. Sequence analysis of the DNA amplicon revealed a 100% homology with the *M. chelonae/ abscessus* group. Even if the classification of the memebrr of this group is still on updating, to the best of our knowledge, this is the first report of *M. chelonae/abscessus* member infection in a Nile crocodile species.

Keywords: ***Mycobacterium*** *chelonae/abscessus*; **crocodile**; **mycobacteriosis, Non-tuberculous mycobacteria**

1. Background

In reptiles, the major causes of disease and mortality are bacterial, fungal, parasitic, and algal agents (1). The Genus *Mycobacterium* includes acid fast, aerobic, non-spore forming, non motile bacteria with wide variations in host affinity and pathogenic potential (2). This genus comprises over 170 species differentiated into the mainly groups of *Mycobacterium tuberculosis* complex (MTC), and mycobacteria other than *tuberculosis* (MOTT) also named non-tuberculous mycobacteria (NTM), that are ubiquitous in the environment (3).

Spontaneous mycobacterial infections have frequently been reported in a wide variety of reptiles, including snakes, turtles, lizards, and crocodiles (1, 4). In particular, crocodiles develop systemic granulomatous inflammation in tegumentary, respiratory and gastrointestinal systems (5). Captive wild animals are more likely to harbour bacterial infections. In fact, reptiles are often household pets or zoo guests, and they might be a source of infection for owners, zoo personnel or veterinarians.

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Moreover, since these species are mostly asymptomatic, they could represent a high potential risk for humans (6). Reptiles during NTM infections develop granulomatous inflammation (1) and many outbreaks in reptiles have been reported in the last years in different countries, including Italy (67). *Mycobacterium spp*, or *Mycobacterium*-like lesions have been previously described in six species of crocodilians (8), mainly due to the aquatic habitat favourable to the pathogen and to the exposure with contaminated food harboring mycobacteria. The last case report in crocodiles described an infection caused by *M. szulgai* in a south Korean *Crocodylus johnstoni* (5).

To the best of our knowledge, this is the first report of an isolation of a member belonging to *Mycobacterium chelonae/abscessus* group in the Nile crocodile (*Crocodylus niloticus*) showing generalized and multiorganic granulomatous lesions.

2. Case Report

A 40 years old male Nile crocodile (*Crocodylus niloticus*) died in the Reptile House of Perugia (Central Italy), without any clinical signs. The reptile arrived in Italy in 1994 from a circus company according to CITES (the Convention on International Trade in Endangered Species of Wild Fauna and Flora) and used to live with two other crocodiles of the same species.

The died crocodile was delivered to Istituto Zooprofilattico Sperimentale dell'Umbria e delle Marche "Togo Rosati" to perform the post mortem examination.

3. Methods

After necropsy procedures were completed, representative tissue samples were collected from organs with pathologic changes for bacteriological, mycological and histological investigations.

The bacteriological examination has been performed from lung, liver, heart and spleen on Blood Agar, MacConkey agar, Mannitol Salt agar, Sabouraud agar. The mycobacteria isolation was performed on selective solid media Stonebrink (Heipha Diagnostika, Germany), BBL Stonebrink TB Medium + PACT (Becton Dickinson, United States), Lowenstein Jensen with glycerol and Lowenstein Jensen without glycerol (Biolife, Italy), after acid and alkaline decontamination, with 1.5% Hexadecylpyridinium chloride (HPC) and 1% NaOH, respectively (9). The slants were incubated in the dark at room temperature, checked daily and suspicious colonies referred to *Mycobacterium* species were submitted to Ziehl–Neelsen (ZN) staining, to assess the acid fastness property. The identification of typical colonies was performed by DNA extraction and PCR assay according to Kulski *et al*. (10). Further characterization was performed by the National Reference Centre for Bovine tuberculosis (Istituto Zooprofilattico Sperimentale Lombardia Emilia Romagna, Brescia), using the MicroSeq 500 16S rRNA-based bacterial ID system. Sequencing analysis was performed in an ABI Prism 3130 genetic analyzer (Applied Biosystems) and data were analyzed by Microseq ID Microbial Identification Software V. 1.0 (Thermofisher Scientific) . Lung, liver, heart and spleen tissue samples were also fixed in 10% neutral buffered formalin for routine histological examination. Samples were embedded in paraffin wax, sectioned at 4 μm and stained with hematoxylin and eosin (HE) and with Ziehl–Neelsen (ZN) staining.

4. Results

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At the post mortem examination the animal showed a good general condition and nutritional status. However systemic granulomatous with visceral involvement was detected. Severe multifocal to coalescing granulomatous pneumonia involving around 90% of lung tissue was observed (Figure 1a). Moreover, from scattered to multifocal granulomas were detected also in heart, liver, spleen and kidney (Figures 1b-d).

Histopathological examination of lung, spleen, heart and liver revealed multifocal to coalescing granulomas showing eterophils in central zone and outer rim of epithelioid histiocytes, multinucleated giant cells and lymphocytes.

The ZN histological staining revealed rare vacuoles containing numerous alcohol-acid resistant bacteria. Moreover, intralesional fungal hyphae were detected.

The microbiological investigations revealed growth only in *Mycobacterium* isolation media: after 10 days we observed few round, shiny and white colonies on Stonebrink slant from lung and spleen (Figure 2). Then, after more 10 days the same colonies became yellow pale and umbelicate (Figure 3). Once ZN staining revealed the presence of alcohol -acid resistance bacilli, the suspected colonies were confirmed by PCR as belonging to *Mycobacterium spp.* by PCR (10).

Finally, 16S rRNA gene sequence analysis revealed that the isolate was 100% identical to *Mycobacterium chelonae/ abscessus* group.

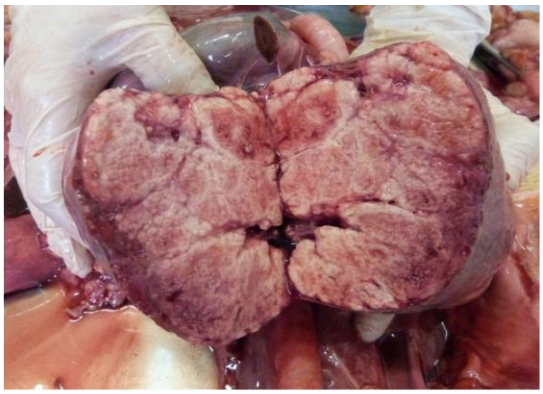

Fig.1a Lung. Multiple coalescing granulomas involving extensively lung tissue.

Fig.1b Heart. Large granulomas involving a left ventricular wall.

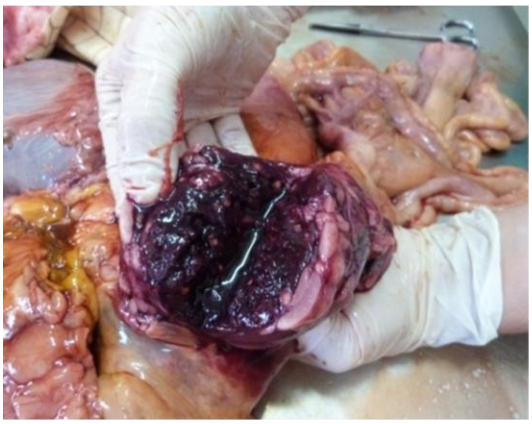

Fig.1c Spleen. Multifocal to coalescing hepatic granulomas.

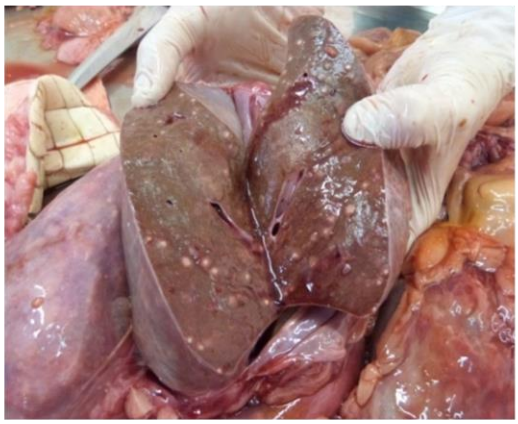

Fig.1d Liver. Multifocal hepatic granulomas.

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Figure 2. Round, shiny and white colonies after 10 days of incubation on Stonebrink Medium .

Figure 3. Yellow pale and umbelicate colony on Stonebrink Medium after 20 days of incubation.

4. Discussion & Conclusions

Mycobacteria are ubiquitous organisms in the environment, and crocodilians are most likely exposed to *Mycobacterium* spp. via horizontal routes such as contaminated water and food (7).

Although confirmed cases of transmission from reptile to humans are rare (8) zoos and reptile houses are an important public health concern because of close contact between mycobacteriosis-susceptible animals and humans, especially veterinarians, zookeepers, and visitors. Isolation of NTM species from organs and tissues presenting granulomas suggests the significance of NTM in causing mycobacteriosis in aquatic animals [3].

Organisms of the *Mycobacterium chelonae/abscessus* group can cause in humans a number of diseases including acute, invasive and chronic infections, which may be difficult to diagnose correctly. Identification among members belonging to this complex is complicated by differentiating at least eleven known species and subspecies and complexity of identification methodologies (11).

This case report describes the *M. chelonae/abscessus* infection in a captive crocodile associated with a systemic mycoses. The pathogenesis of *M. chelonae/abscessus* infection in captive and wild reptiles, as well as the likelihood of animal-to-human transmission, remains to be properly investigated.

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Author Contributions:

M.G.: performed pathology and finalized report, **A**.D.P., S.C., E.M,S.P.: performed histopathology, microbiology and finalized report, M.T., N.D.,E.M., M.S.P.M. : diagnosis and acquisition of samples; M.C., C.S.,M.S. MLP : molecular analyses; A.D.P., P.M:, M.G., N.D. and S.C. wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest: There is no conflict of interests.

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