**Mycobacterium ulcerans** Infection in an Indian Flap-Shelled Turtle (*Lissemys punctata punctata*)

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Abstract. We report an atypical mycobacterial infection in an Indian flap-shelled turtle, *Lissemys punctata punctata*, that died in an aquarium in Japan. At necropsy, the turtle showed multiple white nodules on the capsular surface and parenchyma of various organs such as the liver, spleen, intestine, and lung. Histologically, granulomatous inflammation surrounding a central zone of necrosis was observed. Sections stained by the Ziehl-Neelsen method revealed numerous acid-fast bacilli in the cytoplasm of macrophages and in the central area of necrosis. The organisms were identified as a mycobacterial species by PCR and nucleotide sequence analysis and revealed 98–100% homology to *M. ulcerans*. This is, to our knowledge, the first report of mycobacteriosis due to *M. ulcerans* in a turtle.

Key words: Indian flap-shelled turtle, *Mycobacterium ulcerans* infection, PCR.

*M. ulcerans* is a nonchromogenic slow-growing mycobacterium that causes extensive destruction of skin and soft tissue with the formation of large painless ulcers, usually on the legs or arms, known as Buruli ulcer (BU) in human medicine [20]. Geographically and epidemiologically, the most common sites of occurrence are around rivers, lakes and ponds [20]. With the development of molecular biologic techniques for identification of *M. ulcerans*, the organism has been detected in the environments of Australia and West Africa by using PCR [8, 9]. Some studies have reported BU transmitted from an aquatic sector to humans, and some aquatic bugs or mosquitoes are suspected of the vectors involved [1–3, 6, 8, 10, 14]. This disease affects people in about 32 countries, mainly tropical and semitropical areas, and is seen as a serious problem in the West African countries of Benin, Côte d’Ivoire and Ghana. Temperate areas of Australia and Papua New Guinea are also major endemic countries [20]. On the other hand, non-human cases of natural *M. ulcerans* infection have also been reported, almost simultaneously with epidemic periods of BU, from Victoria, Australia, in koalas, ringtail possums, a captive alpaca and a domestic cat [4, 13].

An adult female Indian flap-shelled turtle (*Lissemys punctata punctata*), which had been imported from India and kept at an aquarium for about 3 years, died from an unknown cause. The turtle had been no external signs of infection, although anorexia often was apparent. The turtle was treated with antimicrobial agents, vitamin supplement and transfusion. Although some recovery signs were evident, the turtle died after 35 days of treatment, and was sent for necropsy to our laboratory. Body weight at time of death was 640 g. Macroscopically, multiple white nodules, 1 to 5 mm in diameter, were present on the capsular surface of lungs, liver, spleen and intestines as well as the parenchyma and membranes lining these cavities and the mesenteriolum (Fig. 1). Several white boggy lesions were also observed on the skin. The organs and skin sampled were fixed in 10% formalin, processed, embedded in paraffin, cut into 3.5 μm sections. All sections were stained with haematoxylin and eosin (HE), as necessary, Ziehl-Neelsen and Grocott. Bacterial DNA were extracted from the affective lesions and neighbor areas, and were then stored at –75°C and used for polymerase chain reaction (PCR).

Histopathologically, the white foci detected in the thoracic and abdominal organs were consistent with granulomatous lesions. There were foci of caseous necrosis in the central area of the lesions which were surrounded by giant cells and epitheloid cells, and encapsulated with fibrous tissues in the outermost layer. Some granulomas were also observed in association with lymphocytes and heterophils peripherally to the necrotic focus or layer of epithelioid (Fig. 2). Granulomatous lesions were also present in the parenchyma of the liver, spleen and pulmonary. In the skin lesions, necrosis of the epidermal layer and some superficial necrotic dermatitis with ulceration were observed, but there were no granulomatous lesions associated. By the Ziehl-Neelsen staining method, acid-fast organisms were mainly detected extracellularly in the necrotic center of lesions, and some located intracellularly in the macrophages and multi nuclear giant cells (Fig. 2, inset). In the skin lesions with ulcer there were no acid-fast organisms with Ziehl-Neelsen, but many fungal organisms were observed by Grocott. Fungal hyphae were branching, septate and 5–8 μm in diameter.

For the detection of *Mycobacterium* spp., PCR assays targeting the *rpoB* gene, and the encoding of the β subunit of
RNA polymerase were used to amplify 324 bp of the product [9]. The primers were MF (5'-CGACCACTTCGGCAACCG-3') and MR (5'-TCGATCGGGCACATCCGGGG-3'). Cycling was as follows: denaturation at 94°C for 5 min, amplification for 35 cycles at 94°C for 30 sec, 55°C for 30 sec, and 72°C for 5 min, and a final extension at 72°C for 5 min. The amplified DNA was electrophoresed through a 1.5% TEA agarose gel and stained with ethidium bromide.

Then two-phased PCR directing at repeat sequences IS2404 of M. ulcerans were performed [6]. The first PCR was performed using the primers MUM1 (5'-GGCAGGCTGGCAGATGGCATA-3') and MUM2 (5'-GGCAGTTACTTCACTGCACA-3'), and a 569 bp product was predicted. Cycling was as follows: denaturation at 94°C for 5 min, amplification for 35 cycles at 94°C for 1 min, 66°C for 1 min, and 72°C for 1 min, and a final extension at 72°C for 7 min. The sequences of the second PCR primers were PGP3 (5'-GGCGCAGATCAACTTCGCGGT-3') and PGP4 (5'-CTGCGTGGTGCTTTACGCGC-3'), and a 218 bp product was predicted. The conditions of the second PCR were the same as those of the first. These amplified products were electrophoresed through a 2% TEA agarose gel and stained with ethidium bromide. The results of PCR are shown in Figs. 3 and 4. The purified PCR products derived from the rpo\(\beta\) and IS2404 were used for sequence analysis with Big-Dye® Terminator v3.1 Cycle Sequencing. The sequence data were subjected to BLAST (The Basic Local Alignment Search Tool) search in the NCBI gene bank.

In mammals, such as humans, cats and koalas, the disease generally forms granulomatous skin lesions with ulcers limited to the skin and soft tissue of the body surface [4, 13, 19]. Granulomatous dermatitis of M. ulcerans is characterized by necrosis often containing extracellular acid-fast bacilli (AFB), and sometimes intracellular AFB in inflammatory exudate cells are observed [16, 19].

M. ulcerans is generally suspected in cutaneous transmission in underwater environments or transfer by mosquito bite [8, 10]. In the present case, a superficial necrotizing dermatitis due to fungus was detected; there were no granulomatous lesions or acid-fast bacteria. We did not identify the skin as the portal of entry. This agent might be transmitted via the oral or respiratory route and spread hematogenously or lymphogenously throughout the body and induced systematic infection which was characterized by visceral granuloma formation in the various organs such as the lung, liver, spleen and intestine. In the turtle, some systematic granulomatous disease due to M. chelonae [5, 7, 12, 15] and osteomyelitis due to M. chelonae have been reported [5, 7, 12, 15]. It is difficult to identify the particular pathogen, because most of them may form the histopathological features similar to our case. Therefore, we consider that the PCR assay is useful to identify the true pathogen among Mycobacterium spp. In Japan, a few cases of Buruli-like ulcer in humans caused by M. ulcerans subsp. Shinshuense have been reported [11, 17, 18]. This bacterium is differentiated from M. ulcerans by the presence of genes on pMUM001 and absence of the serine/threonine protein kinase gene MUP011 in addition to IS2404 [11]. Unfortunately, it is impossible to distinguish between the two by the technique we used. Therefore, it remains uncertain whether this turtle was infected with M. ulcerans or M. ulcerans subsp. Shinshuense in Japan.
minimum, the present results proves that species the turtle in the present case can be infected with *M. ulcerans* and develop granulomatous disease.

Indian flap-shelled turtles are imported to Japan and kept not only at zoos or aquaria, but also as pets. Systematic granuloma due to mycobacterium in the turtle has no noticeable symptoms, so it is difficult to make a diagnosis based on appearance. We think many cases would be first discovered at necropsy. Therefore, if turtles are infected with *M. ulcerans*, it should be appreciated that the zookeeper might be infected from the contaminated environment and develop poor healing ulcers. There are still no drugs well applicable to Buruli ulcer, except at a very early stage. Therefore, patients require surgical resection of the lesion as a first choice, sometimes even amputation of a limb. There is a need to elucidate the infection cycle, vectors, and development of treatment agents.

REFERENCES


