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Mycobacterium ulcerans ecovar Liflandii infection in new host in a Cuban crocodile (*Crocodylus rhombifer*)

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CASE REPORT

ABSTRACT

Mycobacterium ulcerans ecovar Liflandii has recently been described as the causative agent of fatal disease in different frog species. This is the first report of this pathogen in a Cuban crocodile (*Crocodylus rhombifer*), which died suddenly in a Hungarian zoo. The authors' findings suggest that *M. ulcerans* ecovar Liflandii does not exclusively infects anurans and highlights the zoonotic risk posed by this emerging pathogen.

KEYWORDS

crocodile, host species, Mycobacterium, Liflandii, ulcerans

INTRODUCTION

Nontuberculous mycobacteria like *Mycobacterium marinum*, *Mycobacterium chelonae* or *Mycobacterium xenopi* have long been recognised as pathogens of fishes, amphibians and reptiles (Bercovier et al., 2001). Mycobacteriosis in poikilothermic animals is mainly characterised by cutaneous or visceral granulomas.

In 2004, a *Mycobacterium ulcerans*-like pathogen was described in African tropical clawed frogs (*Xenopus tropicalis*) with visceral granulomas, ulcerative and granulomatous dermatitis, coelomitis and septicaemia (Trott et al., 2004). The organism was repeatedly isolated and is now considered an ecotype of *M. ulcerans* (Boulanger et al., 2024; Fremont-Rahl et al., 2011; Suykerbuyk et al., 2007; Tobias et al., 2013).

M. ulcerans is the etiological agent of Buruli ulcer and is closely related to *M. marinum*, one of the classical causative agents of fish tuberculosis (Chany et al., 2013; Decostere et al., 2004; Johnson and Hayman, 1999). The zoonotic potential of *M. marinum* has been well documented, most commonly in the form of fish tank granuloma in humans (Slany et al., 2013). *M. ulcerans* has never been reported to cause disease in animals and is considered a waterborne pathogen endemic to the tropical areas of Africa, Australia, Central America and Eastern Asia. Both *M. ulcerans* and *M. marinum* are mycolactone producing mycobacteria (MPM). Mycolactone is a cytotoxic polyketide composed of a lactone core and a fatty acid side chain. Mycolactones produced by *M. ulcerans* strains from different geographic areas are heterogenic. African isolates produce mycolactone A/B, Australian isolates produce mycolactone C, while Asian isolates produce mycolactone D. Genetic evidence suggests that mycolactone is responsible for the unique disease characteristics of Buruli ulcer (George et al., 1999).

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M. marinum and *Mycobacterium pseudoshottsii* produce mycolactone F, whereas *M. ulcerans* ecovar Liflandii strains produce mycolactone E. To date, all reported epizootics caused by *M. ulcerans* ecovar Liflandii have occurred in laboratory research colonies of *Xenopus* sp. in America or imported from America.

Mycobacteriosis in crocodilians was first described in 1997 (Ariel et al., 1997). Mycobacteria were presumed pathogens for 2.5% of observed skin lesions in crocodiles in an Australian study (Buenviaje et al., 1998). In 2010, *Mycobacterium szulgai* was identified as the causative agent of pulmonary mycobacteriosis of a male freshwater crocodile (*Crocodylus johnstoni*) (Roh Y-S et al., 2010).

In 2015, a Cuban crocodile (*Crocodylus rhombifer*) died suddenly in a Hungarian zoo. This is a report on the isolation and identification of *M. ulcerans* ecovar Liflandii from this individual and as such, a new host species. Since both most closely related species, *M. ulcerans* and *M. marinum* are pathogenic for humans, *M. ulcerans* ecovar Liflandii may therefore also represent a potential zoonotic risk (Boulanger et al., 2024).

MATERIALS AND METHODS

In May 2015, a Cuban crocodile died in a Hungarian zoo without any clinical signs of a disease. The animal was captured in Cuba and transported to Hungary in October 1999. Its age at death was estimated at 25 years. The carcass was in normal body condition and no pathological alterations were observed on the body surface. However, gross necropsy revealed multiple visceral granulomas in the liver, lungs and the ovaries (Fig. 1). Tissue samples were taken and submitted to histopathological, bacteriological and molecular biological examinations.

Tissue samples from the liver were fixed in 10% neutral buffered formalin for 24 h at room temperature, embedded in paraffin and cut into 3–4 µm thick sections. Sections were stained with haematoxylin and eosin, auramine-rhodamine stain and by Ziehl-Neelsen method and subsequently examined under a light microscope.

Tissue samples from the liver were submitted for bacterial culture. After homogenisation and decontamination with 5% oxalic acid the suspension was inoculated onto Löwenstein-Jensen, 7H11 solid media and into Middlebrook broth and incubated parallel at 28 and 37 °C. The strain grew after 14 days of incubation only at 28 °C, both in solid and in liquid culture. Colonies were acid-fast, buff-colored with rough morphology.

For molecular identification, DNA was extracted by sonication. In Wilton and Cousins multiplex Mycobacterium assay the strain displayed only the 1080-bp-long *Mycobacterium* specific band (Wilton and Cousins, 1992). 16S rDNA, *rpo*B, *tuf* and *hsp*65 partial gene sequences were determined, IS2404, *esx*A (ESAT-6) and *esx*B (CFP-10) specific PCRs were performed.

Fig. 1. Variably sized granulomas on both the superficial and cut surfaces of liver (A) and lungs (B) of a Cuban crocodile (*Crocodylus rhombifer*) caused by *Mycobacterium ulcerans* ecovar Liflandii

RESULTS

Gross pathological examination raised suspicion of disseminated mycobacteriosis. In the liver, lungs and ovaries, variably sized, flat or slightly raised tan-white foci were observed on both the superficial and cut surfaces.

Histopathological examination revealed the presence of tuberculotic granulomas in the liver. The central areas of the lesions consisted of eosinophilic coagulative necrosis with karyolysis.

The necrotic core was encircled by histiocytes/epithelioid macrophages, lymphocytes and some Langhans-type giant cells. Fibrosis was also observed around older granulomatous lesions. In the Ziehl-Neelsen stained sections numerous acid-fast bacterial shapes were detected either within macrophages or extracellularly in the affected areas (Fig. 2).

The growth characteristics of the isolated strain were consistent with those of *M. ulcerans* ecovar Liflandii. The performed molecular biological tests identified the pathogen as *M. ulcerans* ecovar Liflandii. The obtained 16S rDNA and *rpo*B gene partial sequences did not allow differentiation of the isolate from *M. ulcerans* and *M. marinum*. The *hsp*65 partial gene sequence was identical to that of *M. ulcerans* ecovar Liflandii (GenBank AY500839), but consistently differed from those of *M. ulcerans* and *M. marinum*. The obtained 714-bp-long partial *tuf* gene sequence was identical to the corresponding section of the *M. ulcerans* ecovar Liflandii complete genome sequence (GenBank CP003899), differed from *M. ulcerans tuf* gene sequence at one, while from the *M. marinum tuf* gene sequence at 6 bases. As no *tuf* gene sequence of *M. ulcerans* ecovar Liflandii was previously available in the GenBank, the sequence generated in this study was deposited under accession number MG385265.

In the IS2404-, *esx*A- and *esx*B-specific PCRs, the strain produced the expected 492-, 187- and 217-bp-long PCR products, respectively.

CONCLUSIONS

M. ulcerans ecovar Liflandii has been recognised as a cause of disease outbreaks in laboratory frogs at multiple research institutions in the past 20 years (Boulanger et al., 2024; Suykerbuyk et al., 2007; Trott et al., 2004).

To the authors' knowledge, this is the first report of *M. ulcerans* ecovar Liflandii causing disease in a crocodile, a new host species for this pathogen. Descriptions of the disease in frogs are very similar, with only minor variations among studies (Boulanger et al., 2024; Fremont-Rahl et al., 2011; Suykerbuyk et al., 2007; Trott et al., 2004).

In this case, lesions were only found in the visceral organs and no cutaneous involvement was observed. This finding may be related to specific biological characteristics of *M. ulcerans* ecovar Liflandii. However, this pathogen is classified as an ecovar of *M. ulcerans*. Genomic analyses have suggested that it shares certain features with *M. marinum*, including the presence of a plasmid related to that of *M. ulcerans,* as demonstrated by Tobias et al. based on complete genome sequence of this pathogen (Tobias et al., 2013). *M. marinum* and *M. ulcerans* produce distinctly different diseases, despite their close taxonomic relationship. Buruli ulcer, the disease caused by *M. ulcerans* in humans, is an extracellular infection, characterised by severe tissue destruction, whereas *M. marinum* causes a granulomatous intracellular infection (George et al., 1999). According to the results of Fremont-Rahl et al., the authors also observed acid-fast bacilli both extracellularly and intracellular within macrophages (Fremont-Rahl et al., 2011).

Tissue damage caused by *M. ulcerans* infections develops mainly due to the cytotoxic effect of the produced mycolactone A/B. As mycolactone E, produced by *M. ulcerans* ecovar Liflandii, is two orders of magnitude less potent than mycolactone A/B, this could also contribute to the granulomatous form of the disease observed in crocodile hosts. Mve-Obiang et al. (2005) highlighted that, in addition to

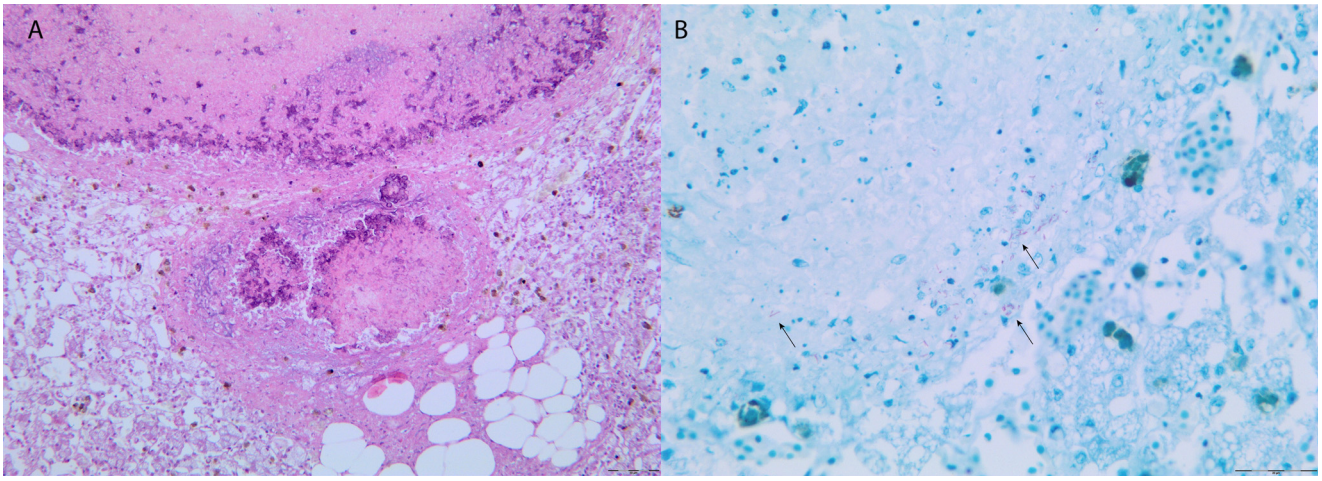

Fig. 2. Granulomas (A) caused by *M. ulcerans* ecovar Liflandii in the liver of a Cuban crocodile (*Crocodylus rhombifer*). The necrotic centre is surrounded by a karyolytic area with some Langhans-type giant cells. B: Acid-fast bacilli visualized by Ziehl-Neelsen staining in the liver tissue

ulcerative, oedematous and plaque forms *M. ulcerans* ecovar Liflandii, disease may also occur in a purely granulomatous form without any skin lesions or signs of oedema.

Presumably, the different manifestations of the disease are not only the consequences of the pathogen's properties, but are also influenced by the development of the host immune system. The exact time of infection could not be determined in our case; however, given the slow progression of mycobacterial diseases, it is presumed that the crocodile was already infected at the time of arrival at the zoo.

An interesting similarity between the observed pathological changes in frogs and the crocodile is that ovaries were involved in all cases (Boulanger et al., 2024; Suykerbuyk et al., 2007; Trott et al., 2004). This finding is of particular significance, as it suggests a potential route for vertical transmission of the pathogen. Since *M. ulcerans* ecovar Liflandii has only been isolated from frog species native to Africa so far, it was assumed that this pathogen is also of African origin, especially since *M. ulcerans* ecovar Liflandii has recently been isolated from a diseased *X. laevis* collected in South Africa (Mve-Obiang et al., 2005). African and Australian *M. ulcerans* isolates lack *esx*A and *esx*B genes and develop more serious diseases. In contrast, *esx*A and *esx*B genes are present in *M. ulcerans* ecovar Liflandii strains and the less pathogenic East Asian, Mexican and South American *M. ulcerans* isolates (Mve-Obiang et al., 2005). This finding would support the possibility that *M. ulcerans* ecovar Liflandii may also be endemic to the American continent. The assumption that the crocodile got infected in Cuba would also support this hypothesis. Moreover, as ESAT-6 and CFP-10 are strong antigens, the presence of *esx*A and *esx*B genes may contribute to the granulomatous response to *M. ulcerans* ecovar Liflandii, as previously suggested by Mve-Obiang et al. in 2005.

Current knowledge regarding *M. ulcerans* ecovar Liflandii remains insufficient to fully define its host spectrum, pathogenicity and zoonotic potential. However, because of the public health importance of both *M. ulcerans* and *M. marinum*, special attention should be paid to the occurrence, geographic distribution and host spectrum of *M. ulcerans* ecovar Liflandii.

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