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NOTE

Mycobacterium gordonae infecting redclaw crayfish *Cherax quadricarinatus*

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ABSTRACT: The redclaw crayfish *Cherax quadricarinatus* (von Martens, 1868) is one of the most commonly exploited freshwater crayfish species worldwide. Redclaw crayfish are susceptible to a number of pathogens but none have been linked to widespread epizootics. Mycobacterial infections have been sporadically reported in crayfish. In the case described, histopathology and bacterial identification confirmed an opportunistic infection caused by *Mycobacterium gordonae* in a hatchery of *C. quadricarinatus* in Israel. Intranuclear inclusion bodies, recorded in cells of the tubular epithelium of the hepatopancreas by histopathology, indicate a co-infection with a viral agent, referable to *C. quadricarinatus* bacilliform virus (CqBV). To the best of our knowledge this is the first description of mycobacteriosis in redclaw crayfish.

KEY WORDS: *Mycobacterium gordonae* · Mycobacteriosis · Redclaw crayfish · *Cherax quadricarinatus* · CqBV

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1. INTRODUCTION

The redclaw crayfish *Cherax quadricarinatus* (von Martens, 1868) (Decapoda: Parastacidae) is one of the most commonly exploited freshwater crayfish species in the world, cultivated mostly in extensive aquaculture systems (Saoud et al. 2013). Native to river catchments in Northern Australia and Southeastern Papua New Guinea, the redclaw crayfish is a non-burrowing and physically robust species that grows rapidly in a wide range of environments (Souty-Grosset et al. 2006). It is ideal for both edible and ornamental purposes, given its broad tolerance to environmental factors and its attractive colours. For these reasons, *C. quadricarinatus* has been translocated to various places in different continents (Gozlan 2010, Lodge et al. 2012, Azofeifa-Solano et al. 2017). In Israel, commercial attempts to farm redclaw crayfish in open systems (Karplus et al. 1998) were not financially sustainable; however, it is cultured in closed systems, mostly for research purposes (Pamuru et al. 2012).

Bacterial infections in crayfish are common and widespread; many of these bacteria are opportunistic agents (Edgerton et al. 2002). In some cases, bacterial infections of crayfish may lead to morbidity and mortalities in both farmed and wild animals, particularly in combination with underlying poor conditions (Edgerton et al. 2002); however, infections have also been reported in asymptomatic crayfish (Longshaw 2011). Alderman et al. (1986) reported a case of nocardiosis in white-clawed crayfish *Austropotamo-*

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bius pallipes possibly caused by bacteria of the genus *Nocardia*. Ahmed et al. (2010) described a case of mycobacteriosis in freshwater red swamp crawfish *Procambarus clarkii*, from the Ibrahimiyah Canal in Egypt, caused by *Mycobacterium fortuitum*.

Mycobacterium gordonae is a slow growing *Mycobacterium* usually found in soil, tap water, and as a laboratory contaminant. *M. gordonae* belongs to a group known as non-tuberculous mycobacteria (NTM) (Covert et al. 1999, Tsankova et al. 2015). Another classification based on Runyon (1959) placed *M. gordonae* as a member of Runyon Group II mycobacteria, belonging to genus *Mycobacterium* (Order *Actinomycetales*, Family *Mycobacteriaceae*). *M. gordonae* is also recognized as an opportunistic microorganism that occasionally occurs in immunocompromised animals and humans (Foti et al. 2009, Freyne & Curtis 2017). Only a few publications describe infections with *M. gordonae* in poikilothermic animals. Sánchez-Morgado et al. (2009) described a case of cutaneous nodular infection in amphibians, caused by *M. gordonae* in a colony of African clawed frogs *Xenopus tropicalis*. *M. gordonae* has also been detected in tropical fish: three spot gourami *Trichogaster trichopterus*, angelfish *Pterophyllum scalare*, cockatoo dwarf cichlid *Apistogramma cacatuoides*, cardinal tetra *Paracheirodon axelrodi* (Lescenko et al. 2003) and guppy *Poecilia reticulata* (Pate et al. 2005). Moreover, *M. gordonae* was also detected in the coldwater goldfish *Carassius auratus* (Pate et al. 2005, Sakai et al. 2005).

In June 2018, the owner of a small-scale recirculating aquaculture system (RAS) hatchery of redclaw crayfish in the center of Israel reported changes in the normal behaviour of some sub-adult and adult individuals, resulting in continued low-grade mortality. Juveniles showed no changes in their normal behaviour or mortality. Symptomatic and asymptomatic crayfish were collected, and histopathology, classical bacteriology and advanced molecular techniques were performed.

2. MATERIALS AND METHODS

2.1. Crayfish sampling

Cherax quadricarinatus broodstock and sub-adults were held in individual net boxes in order to reduce aggressiveness, permitting an easy observation of any anomalous behaviour. During sampling, water temperature was 22°C (±1°C); the weekly water replacement rate was 5%. Asymptomatic juveniles, and symptomatic sub-adults and adults (average weight: 11, 170 and 350 g, respectively) were sampled in 3 sampling events: June, July and November 2018; a total of 19 individuals were collected. Eleven specimens were fixed whole for histopathology examination; 8 specimens were euthanized and dissected for microbiological examination of the hepatopancreas by direct imprints or cultivation on selective media. In order to rule out an infection with *Aphanomyces astaci* (crayfish plague), which can also induce behavioural alteration and mortality, cuticles were also collected from 3 moribund adults.

2.2. Cytology examination

Samples of fresh hepatopancreatic tissue, collected from 3 sub-adult and 5 adult *C. quadricarinatus*, were smeared on glass slides and stained by Ziehl-Neelsen (ZN) staining (0.3% carbol fuchsin and 0.3% methylene blue was used for the staining and hydrochloric ethyl alcohol for the decoloration; Kent & Kubica 1985). Subsequently, glass slides were observed under a light microscope at 40–400× magnification.

2.3. Histopathology examination

The cephalothorax and abdomen of each specimen were injected and fixed in Davidson's fixative for 48 h and post-fixed in 70% ethanol (EtOH). Longitudinal and transversal sections of the abdomen and a longitudinal section of the cephalothorax, comprising the cardial stomach, hepatopancreas, gonads, green gland, ventral nerve chord, heart and gills were obtained from each specimen. Tissues were dehydrated and embedded in Paraplast®, applying standard histological protocols. Thin sections (3 µm) were stained alternatively with Mayer's haematoxylin and eosin (H&E) and ZN, mounted in Eukitt® resin and observed with a Leitz Diaplan microscope at 40–1000× magnification. Digital images were obtained using an integrated Leica MC170HD camera with LAS 4.5.0 (Leica) software.

2.4. Bacterial isolation and characterization

2.4.1. Selective media culturing and ZN staining

Hepatopancreatic tissue was crushed in a mortar with a few drops of deuterium-depleted water (DDW) and decontaminated for 30 min at room temperature

with 1:1 volume of 5% oxalic acid. DDW was added to stop the process, and the sample was centrifuged at 3000 × *g* for 20 min and re-suspended in 5 ml of the supernatant. The sample was then inoculated on solid Löwenstein–Jensen (LJ) and liquid mycobacteria growth indicator tube (MGIT) media and incubated at 30°C until growth was observed. Mycobacterial growth was validated by ZN staining, performed as previously described.

2.4.2. Molecular identification

DNA extraction from the culture and heat inactivation of the bacteria was done by suspending a loopfull of bacteria from LJ medium in 300 µl of water and incubating for 45 min at 95°C in a water bath followed by 15 min incubation in an ultrasonic bath and centrifugation at maximum speed for 5 min. Molecular identification was done using the GenoType *Mycobacterium* CM assay (Hain LifeScience), a PCR targeting the 23S rRNA gene region, followed by reverse hybridization to a nitrocellulose strip coated with probes for different mycobacterial species (Richter et al. 2006). The assay was performed according to the manufacturer's instructions. Briefly, for the amplification reaction, 10 µl of Amplification Mix A (provided with the kit, containing buffer, nucleotides and *Taq* polymerase), 35 µl of Amplification Mix B (provided with the kit, containing salts, specific primers and dye) and 5 µl of the heat-inactivated suspension in a final volume of 50 µl was used. The amplification protocol consisted of 15 min of denaturing at 95°C, followed by 10 cycles of 30 s at 95°C and 2 min at 65°C, an additional 20 cycles of 25 s at 95°C, 40 s at 50°C, and 40 s at 70°C, and a final extension at 70°C for 8 min. The hybridization and detection procedure was performed by mixing 20 µl of the amplification products with 20 µl of denaturing reagent (provided with the kit) and incubating for 5 min in separate wells of a plastic tray. Prewarmed hybridization buffer and membrane strips coated with specific probes were added into each well, and hybridization was done by incubation with shaking for 30 min at 45°C in a TwinCubator instrument (Hain LifeScience), followed by wash for 15 min at 45°C and additional wash for 1 min at room temperature. For colourimetric detection of hybridized amplicons, streptavidin conjugated with alkaline phosphatase and substrate buffer was added and samples were incubated with shaking until bands were clearly visible. The reaction was stopped by rinsing with distilled water, and the result was determined according to the manufacture's interpretation chart.

2.5. Molecular analysis for *Aphanomyces astaci*

Samples of cuticle from the ventral abdomen, telson, proximal joint of the pereiopods, eye-stalk and melanised areas were dissected and fixed in 96% EtOH. Up to 50 mg of mixed cuticles from each individual were collected for total genomic DNA extraction using a QIAamp DNA Mini Kit (Qiagen) according to the manufacturer's instruction (tissue protocol). The extracted DNA was then tested for the presence of *Aphanomyces astaci* by the quantitative TaqMan® minor groove binder (MGB) real-time PCR (Vrålstad et al. 2009) using a CFX96 Real-Time System (BIO-RAD).

3. RESULTS

Imprints of hepatopancreatic tissue, stained by ZN, revealed acid-fast rod-shaped bacteria referable to the genus *Mycobacterium*. Histopathological examination showed melanised haemocytic aggregations (fixed phagocytes) with perivascular cuffing of haemal spaces of the hepatopancreas and encapsulation reactions (Fig. 1A,B); these findings were recorded in 9 out of 11 specimens. In adult and sub-adult specimens, granulomatous-like aggregations were also recorded in the gills (Fig. 1C) and testis (Fig. 1E). In some melanised aggregations, high numbers of acid-fast bacilli (AFB), referable to the genus *Mycobacterium*, were observed using ZN stain (Fig. 1D–F). AFB were evidenced in 7 out of 11 specimens. Moreover, intranuclear inclusion bodies were recorded in cells of the tubular epithelium of the hepatopancreas in all the analysed specimens (Fig. 1H) with various intensity of infection, and were referable to *Cherax quadricarinatus* bacilliform virus (CqBV) (Anderson & Prior 1992, Edgerton 1996), a DNA virus putatively classified in the Family *Nudiviridae* (Bateman & Stentiford 2017). Infected epithelial cells, mostly storage R-cells, had hypertrophied nuclei (2 times the norm) that contained an amorphous pale eosinophilic inclusion (Fig. 1I). In some cases, the development of the inclusion led to chromatin margination and in other cases to remnants of chromatin strands compartmentalizing the inclusion. Infected nuclei tended to have a focal distribution between tubules; some contained several hypertrophied nuclei while adjacent tubules were normal. In some specimens, tubular degeneration with shedding of infected cells was probably preliminary to secondary opportunistic bacterial infection (non-acid-fast coccobacilli) and obliteration of tubular lumen (Fig. 1G). Intranuclear inclu-

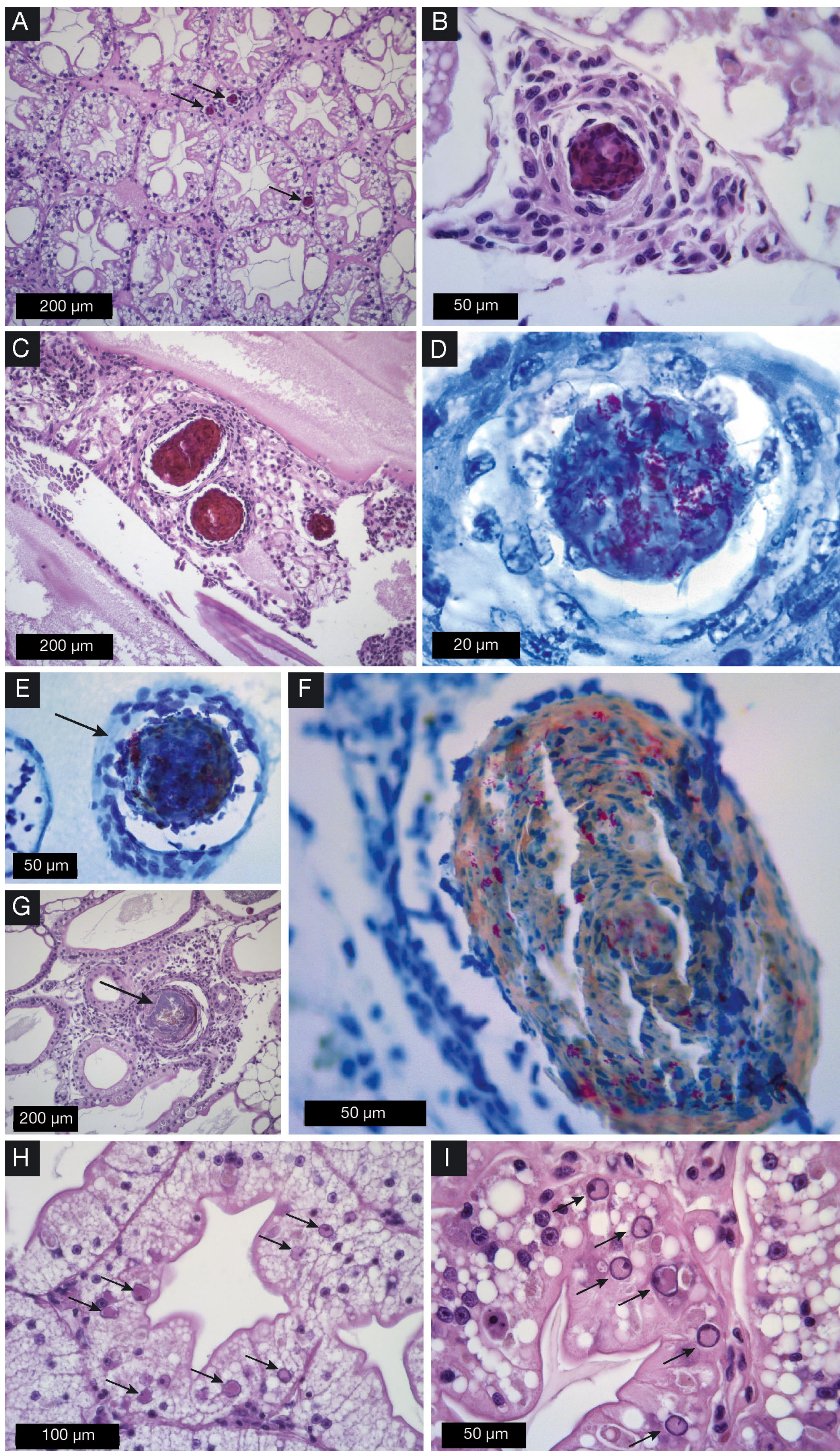

Fig. 1. Histopathological examination of *Cherax quadricarinatus* tissues, showing (A) melanised haemocytic aggregations (arrows) in the haemal spaces of the hepatopancreas (H&E); (B) detail of the perivascular cuffing of the haemal space and encapsulation reaction (H&E); (C) gill tissue with melanised haemocytic aggregation (H&E); (D) detail of haemocytic reaction in the hepatopancreas with evidence of many magenta stained acid-fast bacilli referable to the genus *Mycobacterium* (ZN); (E): haemocytic aggregation in the peritubular connective tissue of the testis with acid-fast bacilli (arrow) (ZN); (F) detail of melanised aggregation in the gill (C) with evidence of magenta stained bacilli (ZN); (G) bacterial proliferation (non-acid-fast bacilli) in the hepatopancreatic tubular lumen with obliteration, melanisation and haemocytic reaction around the affected tubules (arrow) (H&E); (H) intranuclear eosinophilic inclusion (arrows) in R-cells of hepatopancreatic tubule referable to *C. quadricarinatus* bacilliform virus (H&E); (I) detail of the affected nuclei (arrows) with increased size and chromatin margination (H&E). H&E: haematoxylin and eosin; ZN: Ziehl-Neelsen

sion bodies were not recorded in other epithelial tissues of the gill, gut or cuticle. *Mycobacterium* presence in positive culture on LJ medium was verified by AFB presence. The isolate was identified as *M. gordonae*, a slow-growing NTM (Deutsch 2016), with the commercial molecular test GenoType *Mycobacterium* CM. All specimens tested for *Aphanomyces astaci* by quantitative TaqMan MGB real-time PCR were negative.

4. DISCUSSION

We describe an infection of redclaw crayfish *Cherax quadricarinatus* by *Mycobacterium gordonae*, which was identified using histopathology, classical bacteriology and molecular methods. Changes in the normal behaviour of the crayfish (such as uncharacteristic lethargy and a tendency to lie on the side) and continued low-grade mortality were initially suspected as being caused by crayfish plague infection. However, real-time PCR performed on moribund specimens did not reveal *Aphanomyces astaci* DNA. Histopathology examination did not evidence any characteristic aseptate hyphae of oomycetes in crayfish collected during the 3 sampling events, whereas significant presence of intranuclear eosinophilic viral inclusions referable to CqBV were detected only in hepatopancreatic tubules. Although infections by CqBV have not been associated with epizootic mortality before, *C. quadricarinatus* with heavy CqBV infection were reported as being lethargic, unable to right themselves when placed on their back and having a weakened tail-flick response (Longshaw 2011). Moreover, bacteraemia by opportunistic bacteria has been commonly observed in intense infections with CqBV (Edgerton 1996). In the present study, a part of the haemocytic aggregations and melanised nodules in the hepatopancreas and gill sinuses did not reveal AFB in their core. These histopathological changes may indicate a response to bacteraemia by other opportunistic bacteria. Hence, our findings, and the previous descriptions of *M. gordonae* as an opportunist that is occasionally implicated in causing disease among immunocompromised animals and humans (Foti et al. 2009, Freyne & Curtis 2017), describe a case of a co-infection. As is typical in co-infection cases, we can only speculate that the animals were first infected with CqBV, which may have impaired the crayfish immune system. The source of the bacterial infection in this case was not identified; however, *M. gordonae* is likely present in the RAS hatchery. Multiple factors, such as viral co-infection and unsuitable water parameters (e.g. low temperature), are likely to facilitate susceptibility of *C. quadricarinatus* to mycobacteriosis due to *M. gordonae*. As a result of our findings, the owner of the RAS hatchery made the decision to cull the animals and perform a thorough cleaning and disinfection of the hatchery before restocking. As far as we know, this is the first report of *M. gordonae* infecting *C. quadricarinatus*.

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