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1 **SHORT COMMUNICATION**

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3 **Mixed mycobacterial infection in an adult koi carp *Cyprinus carpio* L.**

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14 **Running head**

15 Mixed mycobacteriosis in a koi carp

16

17 **Keywords**

18 *Cyprinus carpio*; *koi*; *Mycobacterium chelonae*; *Mycobacterium abscessus*; PCR-RFLP

19

20 Mycobacteriosis, caused by many different rapidly-growing *Mycobacterium* spp, affects a
21 variety of wild and farmed fish (Decostere, Hermans & Haesebrouck 2004). Affected fish
22 usually show nodular gross lesions in internal organs, anorexia, melanosis and
23 exophthalmia (Frerichs 1993). Diagnosis is usually based on cultural, histopathological
24 and, more recently, molecular investigations. Moreover, piscine mycobacteriosis is a minor
25 zoonosis, causing cutaneous nodular and ulcerative lesions in humans (Wolinsky 1979;
26 Caputo, Fiorella & Orlando 2010), and currently represents a health risk for both fish
27 hobbyists and professional fish farmers (Latha 2004). In particular, *M. marinum*, *M.*
28 *chelonae* and *M. abscessus* are the species most often associated with human diseases
29 (Silcox, Good & Floyd 1981; Shih, Hsueh, Lee, Wang, Yang, Kuo & Luh 1997; Brown-
30 Elliott & Wallace 2002; Haverkort 2003).

31 The koi or fancy carp *Cyprinus carpio* L. var. *koi*, a high value freshwater ornamental fish,
32 is often associated with infectious diseases resulting in fish mortality (Lin, Mao, Guan, Luo,
33 Luo & Pan 2012), concerns over environmental impacts (Matsui, Honjo, Kohmatsu, Uchii,
34 Yonekura & Kawabata 2008), and zoonotic risks (Weir, Rajić, Dutil, Cernicchiaro, Uhland,
35 Mercier & Tuševljak 2012).

36 In July 2012 an adult female koi specimen was sent for *post-mortem* investigations at the
37 Fish Diseases Laboratory of the Istituto Zooprofilattico Sperimentale del Piemonte, Liguria
38 e Valle d'Aosta, Turin. It weighed 2.70 Kg and was 51 cm in length.

39 At necropsy there were four whitish gross lesions suggestive of mycobacteriosis in the
40 spleen (2 to 4 mm), sharply demarcated from the splenic tissue. Moreover, there were
41 numerous atypical nodular lesions in the abdominal cavity (5 to 30 mm). These latter
42 nodules were not well demarcated from surrounding parenchymatous tissues but rather
43 agglomerated the hepatopancreas, ovaries and intestine in an irregular, beige, soft mass
44 (Fig. 1). Duplicates of representative lesions were aseptically sampled from the spleen,
45 ovaries and intestine for histopathological examination, mycobacterial culture and
46 molecular analyses.

47 The tissues for histopathology were fixed in 10% neutral-buffered formalin and processed
48 by standard paraffin wax techniques. Samples were cut into 4±2µm sections and stained
49 with haematoxylin-eosin and Ziehl-Neelsen (ZN) stain. All slides were evaluated
50 microscopically. Diagnosis of mycobacteriosis was based on the observation of multiple
51 granulomas associated with acid-fast rods. Granulomas were detected in all the organs
52 examined (spleen, intestine and ovaries). Granulomas displayed eosinophilic necrotic
53 central areas surrounded by macrophages, epithelioid cells, lymphocytes and plasma cells

54 enclosed by a thin capsule (Fig. 2a-b). Nodules were ZN-positive with mild to moderate
55 numbers of acid-fast bacilli in the necrotic centres and in macrophages (Fig. 2c).

56 Samples for mycobacteria culture and molecular analyses were homogenized and
57 decontaminated by 30 min. immersion in 1.5% cetylpyridinium chloride monohydrate.
58 Swabs from homogenates were inoculated in both Löwenstein-Jensen slant-
59 tubes (VWR®) and Stonebrink's slant-tubes (Microbiol®). Tubes were incubated at
60 $28\pm 2^{\circ}\text{C}$ and $37\pm 2^{\circ}\text{C}$ and examined every two days. ZN staining was used for microscopic
61 examination of all suspected mycobacteria colonies. All swabs from organs were culture-
62 and ZN-positive. A fragment of ~439 bp of the 65-kDa heat shock protein gene (*hsp65*) of
63 the acid-fast isolates was subjected to PCR-restriction fragment length polymorphism
64 (PCR-RFLP) with *Bst*EII and *Hae*III enzymes (MBI Fermentas) (Telenti, Marchesi, Balz,
65 Bally, Bottger & Bodmer 1993). The PCR-RFLP patterns were compared to those reported
66 by Brunello, Ligozzi, Cristelli, Bonora, Tortoli & Fontana (2001). *M. chelonae* was identified
67 from the spleen colonies, and *M. abscessus* from the intestine and ovaries. Co-infections
68 were not detected in the same organ. The PCR-RFLP results were further confirmed by
69 sequencing the *hsp65* gene.

70 Interestingly, although mycobacteriosis has already been reported in various cyprinids
71 (Astrofsky, Schrenzel, Bullis, Smolowitz & Fox 2000; Decostere et al. 2004) including
72 *Cyprinus carpio* L. (Majeed & Gopinath 1983), this report is the first to document infection
73 with two different pathogenic species (*M. chelonae* and *M. abscessus*). The case was
74 also remarkable for both extension and number of lesions in the viscera.

75 Because the koi carp can survive under human care for decades (Tamadachi 1990), the
76 probability of becoming infected with mycobacteria is greater than in other short-living
77 ornamental cyprinids. For this reason, koi breeders should carefully handle fish showing
78 suspicious clinical signs such as tachypnoea, loss of appetite or abnormal pigmentation –
79 especially in aged fish – to avoid the risk of zoonotic infections.

80 Finally, considering the economical importance of the koi industry, and associated
81 international trading, these animals have a potential role as vectors in transmitting
82 mycobacterial organisms from one area to another. The threat of this disease transmission
83 to native species as a result of ornamental fish introduction has been extensively
84 discussed by Passantino, Macri, Coluccio, Foti & Marino (2008).

85 There is no effective cure for mycobacteriosis in fish and there are no vaccines available
86 (Decostere et al. 2004). Removing infected specimens from environments followed by
87 disinfection of contaminated surroundings is usually the only reliable means of control of

88 the disease (Astrofsky et al. 2000). Therefore, a crucial aspect is to prevent its
89 dissemination through biosecurity measures. Chinabut (1999) and Zanoni, Florio,
90 Fioravanti, Rossi & Prearo (2008) also suggested that environmental stress could reduce
91 fish resistance to mycobacteriosis.

92

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96

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152

153 **Figure legends**

154 Figure 1

155 Viscera of the koi carp showing numerous variably sized off-white nodules.

156

157 Figure 2

158 Granulomatous lesions with a central eosinophilic area of necrosis surrounded by
159 inflammatory cells and enclosed by a thin capsule in spleen (a) and ovary (b) (H&E, bar =

160 100 μm). (c) Spleen: Acid fast bacilli within the necrotic core of the granuloma (ZN, bar =

161 10 μm).