

# infection in farmed European tench (L)

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## ACCEPTED MANUSCRIPT

#### 1 *Mycobacterium peregrinum* infection in farmed European tench (*Tinca tinca* L)

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- 12 Keywords: Mycobacterium peregrinum; European tench; mycobacteriosis; farmed fish

#### 13 Abstract

14 This work is the first description of *Mycobacterium peregrinum* as an etiological 15 agent for mycobacteriosis in farmed fishes. We report the mycobacterial infection in 16 farmed European tench (Tinca tinca L) which was confirmed by culture, molecular 17 identification methods (PCRs aimed at 16S rRNA, rpoß and hsp65 sequencing), and 18 histopathology. Since M. peregrinum infection has been described in humans, their 19 clinical significance in fishes should be considered of healthcare interest. With this case 20 report, we also show that a multidisciplinary approach was needed to overcome difficulties associated to diagnosis of piscine mycobacteriosis. 21

#### 22 1. Introduction

Mycobacteriosis of fish is a generic term used to designate a typically subacute to chronic progressive disease caused by different *Mycobacterium* species, being *Mycobacterium marinum*, *M. fortuitum*, *M. chelonae* and *M. abscessus* (Kusunoki and Ezaki, 1992; Decostere et al., 2004) the most commonly identified. However, some new mycobacterial species, such as *M. chesapeaki*, *M. shottsii*, *M. montefiorense* and *M. haemophilum*, among others, have also been implicated as responsible of mycobacterial infections in fish (Levi et al. 2003; Rhodes et al. 2003; Whipps et al., 2007).

Mycobacteriosis is common in marine and freshwater fish-species worldwide, 30 31 including either ornamental fish (Pate et al., 2005; Beran et al., 2006), research facilities 32 (Kent et al., 2004), wild fish (Diamant, 2001; Poort et al., 2006) and cultured fishes as 33 sea bass, striped bass, seabream, snakehead, channel catfish, turbot and Atlantic salmon 34 (Knibb et al., 1993, dos Santos et al., 2002, Brocklebank et al., 2003), where the disease 35 usually produces mortalities between 2-10% (Dos Santos et al., 2002; Ghittino et al., 36 2003). The most important species involved in mycobacteriosis of fish are also zoonotic 37 bacteria responsible of human cutaneous infections associated with manipulation of 38 infected fish or contaminated water (Decostere et al., 2004). The occurrence of human 39 infections with other non-tuberculous mycobacteria (NTM), as M. smegmatis, M. 40 peregrinum and M. septicum, has also increased during the last years (Brown-Elliot & 41 Wallace 2002). Recently *M. peregrinum* has been implemented as a cause of disease in 42 zebrafish research facilities (Kent et al., 2004), and this is the first description of 43 infection by this bacterium in farmed fish (European tench).

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Page 3 of 20

#### 45 **2. Material and methods**

#### 46 *2.1. Farm and animals of the study*

This study was carried out in European tenches (*Tinca tinca* L.) from a farm located in western Spain, with a previous history of low mortality averages (approximately 4% per year). Mortalities occurred generally in adult fish over 500 g, and post-mortem examination of dead fish in the farm revealed the presence of numerous granulomas. European tenches (n = 14) of 750-1000 g and about 300 mm long were captured and sent to the laboratory for microbiological analysis.

Tenches were euthanized with tricaine methanesulfonate (MS-222), necropsied under aseptical conditions and examined for lesions. Ten of the tenches examined presented clinical signs suggestive of systemic mycobacteriosis with external dermal ulcers and internal granulomatous-like lesions, mainly in spleen, liver and kidney. Four tenches were apparently healthy and no clinical signs or lesions were observed. Samples from liver (hepatopancreas), kidney and spleen were taken from affected tenches for histopathological and microbiological (culture and PCR detection) study.

60 *2.2. Histopathology* 

Tissues were fixed in 10% neutral-buffered formaline, embedded in paraffin, cut in 4-µm sections, and stained with haematoxylin-eosin (H&E) and Ziehl-Neelsen's acid fast stains. Histological diagnosis of mycobacteriosis was based on the observation of multiple granulomas in visceral organs and the acid-fast bacilli load in affected tissues.

65 *2.3. Culture* 

66 Auramine staining of smears from tissue samples was performed by the method 67 of Smithwick (1976), and observed by transmission fluorescence microscopy. Tissue

5

68 samples with granulomatous-like lesions from each animal were pooled, homogenized 69 in sterile distilled water, decontaminated with 0.35% hexadecylpyridinium chloride for 70 30 minutes (Corner & Trajstman, 1988), centrifuged at 3.500 r.p.m. (1068 x g) for 30 71 min, and sediments cultured onto selective media (0.2% pyruvate-enriched Löwenstein-72 Jensen and Middlebrook 7H10). Culture media were incubated aerobically at 30°C and 73 checked weekly during 4 weeks for growth.

74 Before decontamination, samples were also cultured for detection of common 75 bacterial fish pathogens onto Columbia blood agar plates (bioMérieux España S.A.) and 76 incubated for 72 hours at 30°C.

77 2.4. Molecular diagnosis

78 Samples were simultaneously processed for DNA extraction following the 79 method of Casas et al. (1995). The identification of isolates from culture media as genus 80 Mycobacterium was determined by two PCR-assays based on the amplification of the 81 16S rRNA and *rpoB* genes; whereas the identification from DNA directly from infected 82 tissues was determined by amplification of *rpoB* gene. In the first reaction, the 83 amplification targets a 1030 bp DNA product specific of 16S rDNA Mycobacterium 84 spp. by using the primers Mycgen-F and Mycgen-R (Table 1) and the amplification 85 conditions described by Boddinghaus et al. (1990). In the second reaction, the primers 86 M-5 and RM-3 were used to amplify a sequence of 136 bp DNA fragment belonging to 87 the *rpoB* gene from NTM following the amplification conditions described by Kim et 88 al. (2004). In both cases, a PCR mixture (50 µl) containing DNA template (50 to 70 ng 89 of chromosomal bacterial DNA or 10 µl of DNA extracted from bacterial suspensions 90 or fish granulomes), 2 mM MgCl<sub>2</sub>, 1 µM of each primer, 0.25 mM of each

91 deoxynucleotide triphosphate and 1.5 U of DNA polymerase (Biotools; B & M 92 Laboratories S.A., Madrid, Spain) in 1x reaction buffer was assembled. The amplicons 93 were visualized by electrophoresis on a 2% agarose gel stained by ethidium bromide and 94 illuminated with UV light. 95 The identification of isolates to species level was carried out by PCR 96 amplification and further sequencing of the 16S rRNA and the 65 KDa heat-shock 97 protein (hsp) genes. The amplification of 1500 bp of the 16S rRNA gene was performed 98 using universal primers (Table 1). Also, a 439 bp fragment of hsp65 was amplified with the primers Tb11 and Tb12 described by Telenti et al. (1993). 99 Amplicons were purified with the Qiaquick PCR Purification kit (Quiagen 100 101 GmbH, Hilden, Germany) and both strands of 16S rRNA and hsp65 genes were 102 sequenced with the DyeDeoxy (dRhodamine) Terminator Cycle Sequencing kit in an 103 automatic ABI Prism 373 DNA sequencer (Applied Biosystems) (C.I.B. Sequencing 104 Facilities, Madrid). The DNA sequences of the clinical Mycobacterium isolates were 105 compared with those available in the GenBank/ **EMBL** databases 106 (http://www.ncbi.nlm.nih.gov) using the BLAST software 107 (http://www.ncbi.nlm.nih.gov/BLAST).

#### 108 **3. Results and discussion**

109 Ten out of the fourteen fishes received showed macroscopic lesions in internal 110 organs compatible with mycobacteriosis (Fig. 1A). The histopathological study was 111 focussed in liver (hepathopancreas), kidney and spleen where we found the most 112 important microscopical findings, although the hepathopancreas always exhibited the 113 most severe lesions.

114 We observed several histopathological findings: 1) initial granulomas composed 115 by numerous grouped macrophages in the central area surrounded of lymphocytes and plasma cells (Fig. 1B). Ziehl-Neelsen stain of these samples revealed the presence of a 116 117 great number of acid-fast bacilli within macrophages (Fig. 1D); and 2) late granulomas 118 composed of multiple coalescing granulomas characterised by eosinophilic necrotic 119 central area surrounded by multinucleated giant cell in scarce number, macrophages, 120 lymphocytes and plasma cells (Fig. 1C). In some cases the lesions were so intense that 121 the architectural disorganization made organ recognition difficult. In addition, some 122 examined fishes displayed a wide portal-portal bridging fibrosis associated with intense 123 infiltration of lymphocytes, plasma cells and macrophages; this process originated a 124 parenchyma collapse given place to small nodular foci of hepatocytes (micronodular 125 cirrhosis). The hepatic parenchyma displayed also a severe hydropic degeneration of 126 hepatocytes. The renal interstitium presented a moderate to severe chronic infiltration of 127 round cells, mainly lymphocytes, plasma cells and macrophages, especially in those 128 cases where granulomatous lesions were found. These changes could be due to the 129 increase in the hematopoietic capacity of the kidney in response to the infection. All 130 these pathological findings suggest that these granulomatous lesions had been induced

#### 131 by a *Mycobacterium* infection.

132 Although histological lesions were strongly indicative of mycobacteriosis, 133 culture was unsuccessful in most samples analyzed, and mycobacteria were isolated in 134 both Löwenstein-Jensen and Middlebrook 7H10 agars from only two tenches (isolates 135 TCI 1 and TCI 2). Both isolates were identified as *Mycobacterium* spp. by PCR aimed 136 at the 16S rDNA (Fig. 2A). On the other hand, the 136 bp amplification product of 137 Mycobacterium NTM rpoB gene (Fig. 2B), were obtained from tissue homogenates of 138 four other affected tenches. No other bacteria were isolated from primary cultures onto 139 Columbia blood agar plates.

In this study, we used a molecular identification approach to overcome the 140 141 difficulties associated to biochemical identification of mycobacteria that is lengthy, 142 difficult to perform and can lead to inconclusive results. The 16S rRNA sequence of the 143 clinical isolate TCI 1 has been deposited in the GenBank/EMBL database under the accession number AM884581. Comparative analysis of the 16S rRNA sequences 144 revealed that both *Mycobacterium* clinical isolates were identical, displaying 100% 145 similarity with the type strain of *Mycobacterium peregrinum* CIP 105382<sup>T</sup> (accession 146 147 number AY457069). As M. peregrinum has similar 16S rRNA sequences to M. 148 septicum (which only 4 substitutions across 1483 bp), the identification of the clinical 149 isolates was also confirmed by sequencing of the hsp65 gene. Sequence analysis of this 150 gene confirmed the identity of the clinical isolates, displaying 99% similarity with the 151 hsp65 sequence of M. peregrinum isolate B1285 (GenBank/EMBL accession number 152 AY379072) of this microorganism. The two point mutations (at nucleotides 617 and 153 800) found in our isolates (GenBank/EMBL accession number EU156064, isolate TCI

154 1) have been previously described in *M. peregrinum* (Ringuet et al., 1999).

155 Although *M. peregrinum* is a fast-growing mycobacteria (Kusunoki and Ezaki, 1992), the unsuccessful culture of this microorganism on the media routinely used for 156 157 the isolation of *Mycobacterium* spp. has also been observed previously. Thus, M. 158 peregrinum was isolated on Middlebrook 7H10 agar from only 9% of experimentally 159 inoculated zebrafish (Watral and Kent, 2007) and in fishes infected by other 160 micobacterial opportunistic pathogen (Watral and Kent, 2007; Whipps et al., 2003). 161 These findings are in agreement with the lack of equivalence in some piscine 162 mycobacterial infections between the presence of granulomatous lesions, the presence 163 of acid-fast bacilli and the culture growth. Similar results have been also described in 164 mycobacteriosis in birds caused by M. peregrinum (Vitali et al., 2006). M. peregrinum 165 could be considered as an opportunistic pathogen that may affect animals under stressful conditions (i.e. higher water temperature, mating season) (Vitali et al., 2006; 166 Watral and Kent, 2007); thus, some animals would survive the clinical infection 167 168 although the granulomatous lesions may remain in their internal organs. This fact 169 highlights that an appropriate approach to diagnosis should include histopathology, 170 bacteriology and molecular identification.

*M. peregrinum* has previously been isolated from nodular lesions of cultured marine white shrimp *Penaeus vannamei* (Mohney et al., 1998) and from different ornamental fish species without apparent clinical lesions (Pate et al., 2005). However, there is a lack of knowledge about the importance and impact of *M. peregrinum* infections on both wild and cultured-fish populations. There is only one report of mycobacterial outbreaks in zebrafish research facilities by *M. peregrinum* (Kent et al.,

177 2004), although in this outbreak the identification of the etiological agent as *M*.
178 peregrinum was not unambiguously confirmed (Kent et al., 2004). In our study, *M*.
179 peregrinum was isolated and accurately identified from tenches with visible
180 granulomas, being therefore the first report of mycobacteriosis in farmed fishes
181 implicating *M. peregrinum*. The multidisciplinary approach used in this study allowed
182 us to overcome difficulties associated to diagnosis of piscine mycobacteriosis, and a
183 combination of molecular identification yielded a precise diagnosis.

184 Mycobacterial infections in humans are associated with exposure to fish or contaminated water. Over the last decade, a small but increasing number of sporadic 185 186 human infections associated with *M. peregrinum* have been reported (Ishii et al., 1998, 187 Pagnoux et al., 1998, Rodriguez-Gancedo et al, 2001, Koscielniak et al., 2003, Short et 188 al., 2005, Sakai et al., 2005). Most of these infections occur in immune compromised 189 persons, but unusual M. peregrinum infections in non immune-compromised persons 190 have also been described (Ishii et al., 1998). European tench is a cyprinid farmed fish 191 species cultured for human consumption. Although the transmission of *M. peregrium* to 192 man through the consumption of contaminated fish is very unlikely, the improper 193 manipulation of *M. peregrinum* infected fish may represent a potential risk for fish 194 farmers or fish processors, as well as it has been described for other zoonotic bacterial 195 fish pathogens (Ghittino et al., 2003). Tenches are also used as live bait for sport 196 activities, and as observed with other mycobacteria, this fish infection could represent a 197 potential source of infection for other wild-fish species (Kane et al., 2007), as well as 198 for humans in the course of recreational water related activities (Ang et al., 2000; 199 Decostere et al., 2004; Primm et al., 2004).

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Table 1. Primers used in this study.

Primer	Sequence (5'→3')	Gene	Size (bp)	Target
Mycgen-F	AGAGTTTGATCCTGGCTCAG	1(0 DNA	1020	
Mycgen-R	TGCACACAGGCCACAAGGGA	105 IKNA	1030	1030 Mycobacterium spp identification
M-5	GGAGCGGATGACCACCCAGGACGTC	гроβ	126	136 Non-tuberculous mycobacteria identification
RM-3	CAGCGGGTTGTTCTGTCCATGAAC		136	
ARI	GAGAGTTTGATCCTGGCTCAGGA	1(0 D)14	1500	T1 / C / I 1
РН	AAGGAGGTGATCCAGCCGCA	16S rKNA	1500	Identification at species level
Tb11	ACCAACGATGGTGTGTCCAT	1 (5	4.4.1	
Tb12	CTTGTCGAACCGCATACCCT	hsp65	441	Identification at species level

#### Legends to Figures

**Fig. 1.** Gross lesions: (A) Multiple whitish nodules in the hepatopancreas. Histopathological findings: (B) Initial granuloma composed of numerous macrophages in the central area. Kidney. H&E 10x; (C) Late granuloma characterized by necrotic centre around with multinucleated cells, great number of macrophages and lymphocytes. Hepatopancreas. H&E 4x. (D) Initial granuloma with numerous intracytoplasmatic acid-fast bacilli within macrophages and multinucleated cells (arrows). Kidney. Ziehl-Neelsen 20 x.

**Fig. 2.** Ethydium bromide-stained agarose gel electrophoresis of the PCR products generated from mycobacterial clinical culture isolates and granulomatous lesions from tench using the PCR assay against the 16S rRNA (panel A; amplicon size of 1030 bp) and the *rpoB* (panel B; amplicon size of 136 bp) genes. (A) line 1, molecular size marker (100-bp ladder); line 2, *Mycobacterium* positive control, *M. marinum* CECT 3024<sup>T</sup>; line 3, negative control; lines 4-6, clinical isolate TCI and TC2 from different media. (B): line 1, molecular size marker (100-bp ladder); line 2, *Mycobacterium* positive control, *IIII* and TC2 from different media. (B): line 1, molecular size marker (100-bp ladder); line 2, *Mycobacterium* positive control, *M. marinum* CECT 3024<sup>T</sup>; line 3, negative control; lines 4-6, clinical isolate TCI and TC2 from different media. (B): line 1, molecular size marker (100-bp ladder); line 2, *Mycobacterium* positive control, *M. marinum* CECT 3024<sup>T</sup>; line 3, negative control; lines 4-7, tissue homogenates of affected tenches.





