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

2 A. Aranaz^a, A. Gibello^{a,*}, J. Álvarez^a, A. I. Mata^a, A. Rodríguez^b, C. Fallola^c, J.F.
3 Fernández-Garayzábal^a, L. Domínguez^a

4 ^a Laboratorio de Vigilancia Sanitaria Veterinaria (VISAVET), Departamento de
5 Sanidad Animal, Facultad de Veterinaria, Universidad Complutense de Madrid,
6 Avda. Puerta de Hierro s/n, 28040 Madrid, Spain

7 ^b Departamento de Medicina y Cirugía Animal, Facultad de Veterinaria, Universidad
8 Complutense de Madrid, Avda. Puerta de Hierro s/n, 28040 Madrid, Spain

9 ^c Dirección General de Medio Ambiente, Junta de Extremadura, Badajoz, Spain

10 * Corresponding author. Tel.: + 34 91 3943900; fax: + 34 91 3943908.

11 E-mail address: gibelloa@vet.ucm.es

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, rpo β 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
21 difficulties associated to diagnosis of piscine mycobacteriosis.

22 1. Introduction

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

30 Mycobacteriosis is common in marine and freshwater fish-species worldwide,
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).

44

45 **2. Material and methods**

46 *2.1. Farm and animals of the study*

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

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

60 *2.2. Histopathology*

61 Tissues were fixed in 10% neutral-buffered formaline, embedded in paraffin, cut
62 in 4- μ m sections, and stained with haematoxylin-eosin (H&E) and Ziehl-Neelsen's acid
63 fast stains. Histological diagnosis of mycobacteriosis was based on the observation of
64 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

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 Boddinhaus 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 MgCl₂, 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
99 the primers Tb11 and Tb12 described by Telenti et al. (1993).

100 Amplicons were purified with the Qiaquick PCR Purification kit (Quiagen
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 (hepato-pancreas), kidney and spleen where we found the most
112 important microscopical findings, although the hepato-pancreas 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
116 plasma cells (Fig. 1B). Ziehl-Neelsen stain of these samples revealed the presence of a
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.

140 In this study, we used a molecular identification approach to overcome the
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
144 accession number AM884581. Comparative analysis of the 16S rRNA sequences
145 revealed that both *Mycobacterium* clinical isolates were identical, displaying 100%
146 similarity with the type strain of *Mycobacterium peregrinum* CIP 105382^T (accession
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,
156 1992), the unsuccessful culture of this microorganism on the media routinely used for
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 (Watrall and Kent, 2007) and in fishes infected by other
160 mycobacterial opportunistic pathogen (Watrall 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
166 stressful conditions (i.e. higher water temperature, mating season) (Vitali et al., 2006;
167 Watrall and Kent, 2007); thus, some animals would survive the clinical infection
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.

171 *M. peregrinum* has previously been isolated from nodular lesions of cultured
172 marine white shrimp *Penaeus vannamei* (Mohney et al., 1998) and from different
173 ornamental fish species without apparent clinical lesions (Pate et al., 2005). However,
174 there is a lack of knowledge about the importance and impact of *M. peregrinum*
175 infections on both wild and cultured-fish populations. There is only one report of
176 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
185 contaminated water. Over the last decade, a small but increasing number of sporadic
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. peregrinum* 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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316

Table 1. Primers used in this study.

Primer	Sequence (5'→3')	Gene	Size (bp)	Target
Mycgen-F	AGAGTTTGATCCTGGCTCAG	16S rRNA	1030	<i>Mycobacterium</i> spp identification
Mycgen-R	TGCACACAGGCCACAAGGGA			
M-5	GGAGCGGATGACCACCCAGGACGTC	rpoβ	136	Non-tuberculous mycobacteria identification
RM-3	CAGCGGGTTGTTCTGTCCATGAAC			
ARI	GAGAGTTTGATCCTGGCTCAGGA	16S rRNA	1500	Identification at species level
PH	AAGGAGGTGATCCAGCCGCA			
Tb11	ACCAACGATGGTGTGTCCAT	<i>hsp65</i>	441	Identification at species level
Tb12	CTTGTCGAACCGCATAACCT			

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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 intracytoplasmic acid-fast bacilli within macrophages and multinucleated cells (arrows). Kidney. Ziehl-Neelsen 20 x.

Fig. 2. Ethidium 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^T; 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^T; line 3, negative control; lines 4-7, tissue homogenates of affected tenches.



