First isolation of *Mycobacterium* spp. in *Mullus* spp. in Turkey

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(Received 19 Mar 2014; revised version 17 Dec 2014; accepted 7 Jan 2015)

Summary

Ichthyozoonotic *Mycobacterium* spp. poses health risks both to fish and humans. In this study, the presence of ichthyozoonotic *Mycobacterium* spp. was investigated in red mullet (*Mullus barbatus barbatus*) and surmullet (*Mullus surmuletus*), widely caught species in the Mediterranean and the Aegean Sea. A total of 208 fish samples, provided from fishermen of Mersin province (Turkey) were studied. Using conventional methods, *Mycobacterium* spp. was isolated and identified at the genus level by PCR and at the species level by PCR-RFLP. Thirteen *Mycobacterium* spp. were detected in 13 (6.25%) fish samples. Four mycobacteria were identified as *M. genavense*, three as *M. fortuitum*, three as *M. scrofulaceum*, one as *M. marinum*, one as *M. vaccae* and one as *M. aurum*. No signs of mycobacteriosis were observed in fish samples. Findings of this study can contribute to future studies of onichthyozoonotic *Mycobacterium* spp. in seafood.

Key words: Fish disease, Food safety, *Mycobacterium* spp., Red mullet (*Mullus barbatus barbatus*), Surmullet (*Mullus surmuletus*)

Introduction

Atypical mycobacteria are commonly found in nature and known as "nontuberculous *Mycobacterium*" (NTM) or "environmental mycobacteria". If transmitted by water and aquatic organisms, some *Mycobacterium* species can become infectious to humans, fish and many other animals (Nichols *et al*., 2004; Jacobs *et al*., 2009).

In cases of immune deficiencies, NTM can cause several infections in humans, mostly in soft tissues and skin (Sanders *et al*., 1995). Seafood related environmental mycobacteria mostly pose risks to fish handlers, aquarium hobbyists (Decostere *et al*., 2004), and even raw fish consumers.

Certain environmental *Mycobacterium* species can cause "fish mycobacteriosis", which is a contagious and chronic disease. External symptoms may include emaciation, stunted growth, exopthalmia, dermatitis, and ulcer. It is also characterized by internal symptoms of small tubercules, typically apparent in the spleen, liver and head kidney. Fish mycobacteriosis is a widely distributed infection reported in more than 167 fish species and can be seen in all freshwater, saltwater and ornamental fish (Austin and Austin, 2007; Jacobs *et al*., 2009). Although *Mycobacterium* in saltwater fish has been investigated in many countries (Perez *et al*., 2001; Dos Santos *et al*., 2002; Rhodes *et al*., 2004), only one case has been reported in Turkey so far (Korun *et al*., 2005).

In Turkey, the demersal species red mullet (*Mullus barbatus barbatus*, Linnaeus, 1758) and surmullet (*Mullus surmuletus*, Linnaeus, 1758) are commercially important. They are well appreciated and widely consumed, and are caught by trawlers in the Mediterranean and Aegean regions.

Taking into account that *Mycobacterium* infections pose risks to both fish and human health, this study was carried out to investigate the presence of environmental *Mycobacterium* spp. in red mullet and surmullet caught from three different sites along the Mersin coastline (Eastern Mediterranean), a major fishing area and fishing harbour for these two species in Turkey.

Materials and Methods

Fish samples

Fish sampling was performed between September 2009 and October 2010, including autumn, winter, and spring seasons. Summer sampling failed because of the ban put on fishing. Fish samples, obtained from commercial trawlers at three different fishing harbours (Karaduvar-Mersin, Taşucu and Anamur) on the Mersin coastal line (Fig. 1), were transferred to the Fish Diseases Laboratory (Faculty of Fisheries, University of Mersin), following hygiene and cold chain rules. From the total of 208 fish samples, 135 specimens were identified as red mullet (*Mullus barbatus barbatus*) and 73 as surmullet (*Mullus surmuletus*). The mean values (±SD) of fish total length and body weight were determined as 13.80 ± 2.10 cm and 31.67 ± 14.58 g for red mullet and as 15.49 ± 1.70 cm and 45.20 ± 14.52 g for surmullet, respectively.

Prior to the microbiological examination, fish samples were examined internally and externally for the presence of mycobacteriosis (Austin and Austin, 2007).

Isolating mycobacteria by conventional methods

The NaOH modified Petroff method was used to isolate *Mycobacterium* by conventional techniques. A total of 624 homogenates were prepared from skin, muscle and visceral organs of the samples. From each

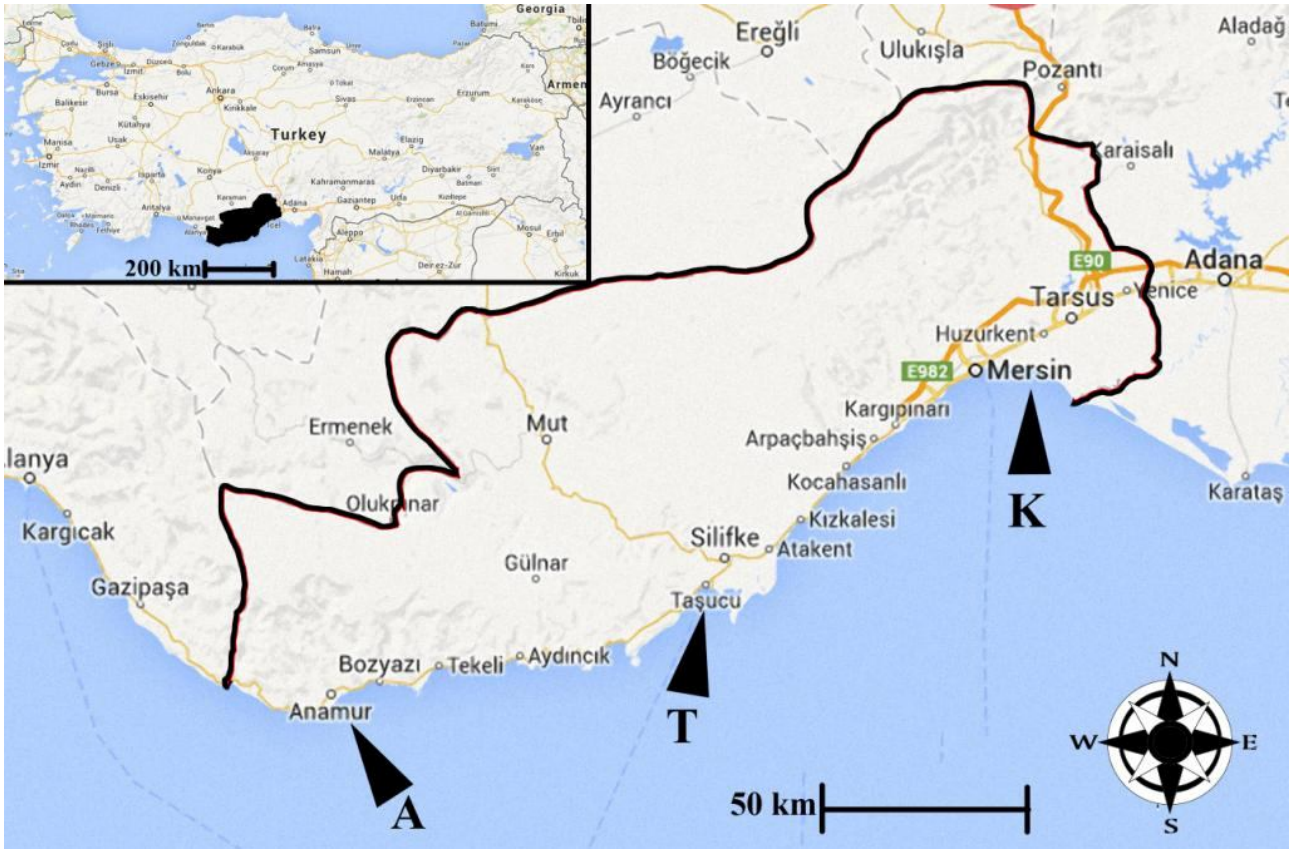

Fig. 1: Map of the fish sampling points (K: Karaduvar-Mersin, T: Taşucu, and A: Anamur)

processed sample, 0.1 ml was inoculated onto a Löwenstein-Jensen (L-J) agar and Middlebrook 7H9 medium (Anonymous, 2009). Incubation was performed at 25°C for 6-8 weeks (Austin and Austin, 2007).

Identification of isolates by polymerase chain reaction (PCR)

Bacterial DNA extraction

A modified rapid method developed by Sajduda *et al*. (2004) was used for DNA extraction of *Mycobacterium* spp. The DNA extraction treatment was applied to the suspected *Mycobacterium* spp. colonies and reference strains isolated on L-J agar. A loopful of bacterium was suspended in 1 ml sterile distilled water. After lysing by heating in boiled water for 20 min, samples were centrifuged at 12000 g for 15 min and supernatant was discharged. The pellet was stirred with vortex for 1 min after adding 200 µL cloroform. By adding 200 µL nuclease-free distilled water it was restirred, and centrifuged at 12000 × g for 15 min. This supernatant was used as DNA template in PCR amplification.

Mycobacterium aurum (DSMZ 6695), *Mycobacterium gordonae* (RSKK 14470), *Mycobacterium chelonae* (RSKK 06064), *Mycobacterium fortuitum* (ATCC 6841) and *M. tuberculosis* (H37Rv) were used as reference strains.

Amplification of hsp65 gene area

For the amplification of the *hsp*65 gene area, 5 µL of the template DNA were added to each reaction tube. The PCR blend consisted of 50 mM KCl, 10 mM Tris-HCl (pH = 8.3), 1.5 mM $MgCl_2$, 10% glycerol, 200 µM from each deoksinucleosid triphospate, 0.5 µM of primers [Tb11 5′-ACCAACGATGGTGTGTCCAT (sense) and Tb12 5′-CTTGTCGAACCGCATACCCT (antisense), TIB Molbiol, Germany] and 1.25 U *Taq* DNA polymerase (Sigma-Aldrich, D-1806 5 U/µL). The PCR programme used in the amplification was as follows: the first denaturation was applied at 94°C for 5 min, afterwards, 45 amplification cycles (1 min at 94°C, 1 min at 60°C, 1 min at 72°C) were applied and awaited at 72°C for 10 min for ultimate elongation. The amplicons were visualized by electrophoresis on a 1.5% agarose gel stained by ethidium bromide and illuminated with UV light (Telenti *et al*., 1993).

Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP)

PCR-RFLP was carried out using the PCR product of 439 bp amplifiers. To cut with *Bst*EII and *Hae*III enzymes (Fermentas, #ER0391, Fermentas GMBH, Germany), 10 µL PCR product was added to the mixture prepared with each 0.5 µL (5 U) enzyme, 2.5 µL enzyme buffer (10 X Buffer-O) and 11 µL nuclease-free distilled water and incubated at 37°C for 4 h. Electrophoresis of cutting products was performed in a 2% agarose gel. Differentiation of mycobacterial isolates at species level was carried out by assessing the patterns formed after the cutting reaction (Telenti *et al*., 1993).

Results

Despite liver paleness in nine of the fish samples, mycobacteriosis signs were not observed in any of the specimens.

Out of 624 samples, suspected *Mycobacterium* spp. colonies were observed in 73 L-J agar tubes. Since bacterial growth on Middlebrook 7H9 broth and L-J agar revealed similar results, only isolates of L-J agar (Fig. 2) were used for further identifications.

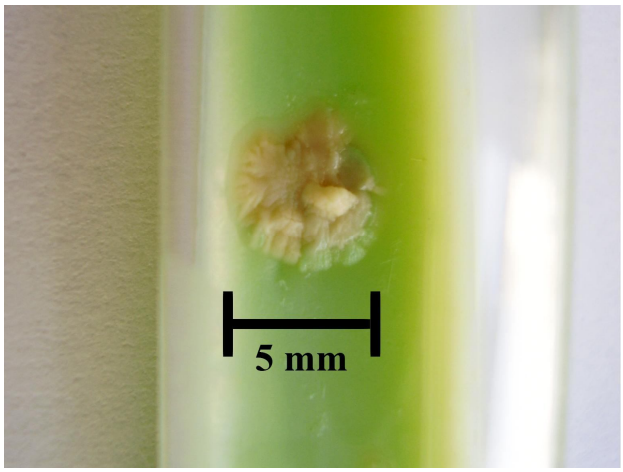

Fig. 2: A colony of *Mycobacterium* sp. on Löwenstein-Jensen agar

Even though no acid-fast rods were detected by ZN staining in any of the tissue homogenates, a total of 22 positive results were obtained on L-J agar isolates (Fig. 3). These isolates were interpreted as suspected *Mycobacterium* spp. colonies and kept in refrigerator at +4°C until the next stage.

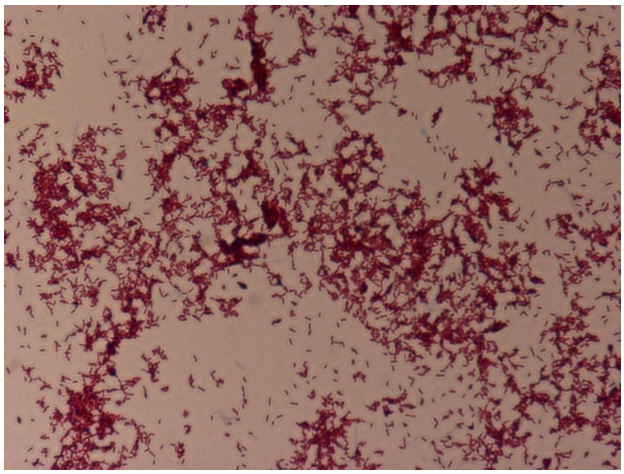

Fig. 3: Acid-fast rods (Ziehl-Neelsen staining, ×1000)

Mycobacterium spp. isolates determinated by primer specific PCR

The presence of band patterns at the length of 439 bp revealed that 13 out of the 22 acid-fast rods (59.1%) were *Mycobacterium* spp. (Fig. 4).

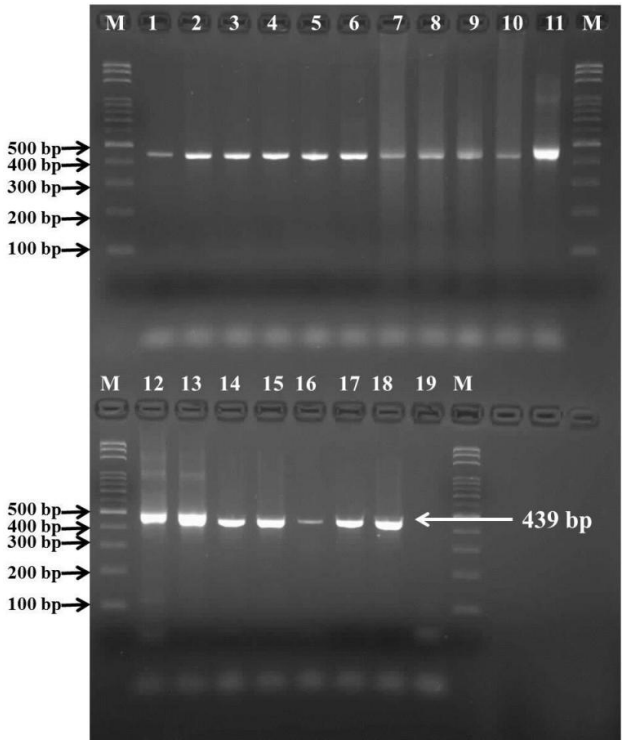

Fig. 4: Electrophoresis image of primer specific PCR. Column M: 100 bp of DNA molecular weight marker (Amresco, 100 bp ladder, K180-250UL), Column 1-13: *Mycobacterium* spp. isolates, Column 14: *M. gordonae* (RSKK 14470), 15: *M. fortuitum* (ATCC 6841), 16: *M. chelonae* (RSKK 06064), 17: *M. aurum* (DSMZ 6695), 18: *M. tuberculosis* (H37Rv), and 19: Negative control

According to *Bst*EII and *Hae*III enzymes and base pair lengths of band patterns, *Mycobacterium* spp. isolates were identified at the species level (Table 1) (Figs. 5 and 6).

Out of 208 *Mullus* spp. samples studied in this work, thirteen mycobacteria isolates were detected in 13 (6.25%) specimens. Of these isolates, 10 (76.9%) were proliferated in *Mullus barbatus barbatus* and 3 (23.1%) in *Mullus surmuletus*. While 12 of these isolates were detected in the skin of the fish samples, one was found in internal organs. As related to seasons, 4 of the 13 isolates were found in the 2009 autumn samples (30.8%), and the remaining 9 isolates were detected in autumn (3 isolates), winter (3 isolates), and spring (3 isolates) samples in 2010. As per fishing harbours, 3 of the 13 isolates were found in samples collected from Anamur (23%), 6 from Taşucu (46%) and the remaining 4 in samples from Karaduvar (31%) harbours. Six different species of the isolates were identified as *Mycobacterium genavense* (4/13), *M. fortuitum* (3/13), *M. scrofulaceum* (3/13), *M. marinum*, *M. vaccae*, and *M. aurum* (1/13, each) (Table 2).

Discussion

Environmental mycobacteria are widespread in nature, especially in water and mud. Although the majority of these rods are saprophyte, some have

opportunistic features in humans, fish, and many other animals. Fish contaminated by these bacteria could be a source of zoonotic risk for human health (Bercovier and Vincent, 2001; Jacobs *et al*., 2009). For this reason, mycobacteria have been studied and detected in many fish species around the world including various wild marine fish species (Diamant *et al*., 2000; Heckert *et al*., 2001; Perez *et al*., 2001; Levi *et al*., 2003; Whipps *et al*., 2003; Rhodes *et al*., 2004, 2005; Jacobs *et al*., 2009; Gauthier *et al*., 2010). In this study, the isolation of

Table 1: Determination of mycobacteria by PCR-RFLP according to the type of enzyme and base pair (bp) lenghts (Telenti *et al*., 1993)

Row	Isolate No.	*Bst*EII enzyme	*Hae*III enzyme	*Mycobacterium* species
1	K[k] 14 S[s]	325 bp, 125 bp	140 bp, 105 bp[b]	*M. genavense*
2	A[a] 13 S	245 bp, 125 bp, 80 bp	155 bp, 135 bp	*M. fortuitum*
3	K 16 S	325 bp, 125 bp	140 bp, 105 bp	*M. genavense*
4	K 18 S	325 bp, 125 bp	140 bp, 105 bp	*M. genavense*
5	A 28 S	245 bp, 220 bp	160 bp, 115 bp, 80 bp	*M. marinum*
6	T[t] 22 I[i]	245 bp, 140 bp, 85 bp	175 bp, 80 bp	*M. aurum*
7	K 42 S	325 bp, 125 bp	140 bp, 105 bp	*M. genavense*
8	A 46 S	439 bp	175 bp, 80 bp	*M. vaccae*
9	T 56 S	245 bp, 220 bp	155 bp, 135 bp, 95 bp	*M. scrofulaceum*
10	T 57 S	245 bp, 220 bp	155 bp, 135 bp, 95 bp	*M. scrofulaceum*
11	T 68 S	245 bp, 125 bp, 80 bp	155 bp, 135 bp	*M. fortuitum*
12	T 70 S	245 bp, 220 bp	155 bp, 135 bp, 95 bp	*M. scrofulaceum*
13	T 72 S	245 bp, 125 bp, 80 bp	155 bp, 135 bp	*M. fortuitum*
Reference strains				
14	RSKK 14470	245 bp, 125 bp, 80 bp	170 bp, 115 bp	*M. gordonae*
15	ATCC 6841	245 bp, 125 bp, 80 bp	155 bp, 135 bp	*M. fortuitum*
16	RSKK 06064	245 bp, 220 bp	160 bp, 60 bp	*M. chelonae*
17	DSMZ 6695	245 bp, 140 bp, 85 bp	175 bp, 80 bp	*M. aurum*
18	H37Rv	245 bp, 125 bp, 80 bp	160 bp, 140 bp, 70 bp	*M. tuberculosis*

[a] Anamur, [k] Karaduvar, [t] Taşucu, [S] Skin, [i] Internal organs, and [b] Base pairs

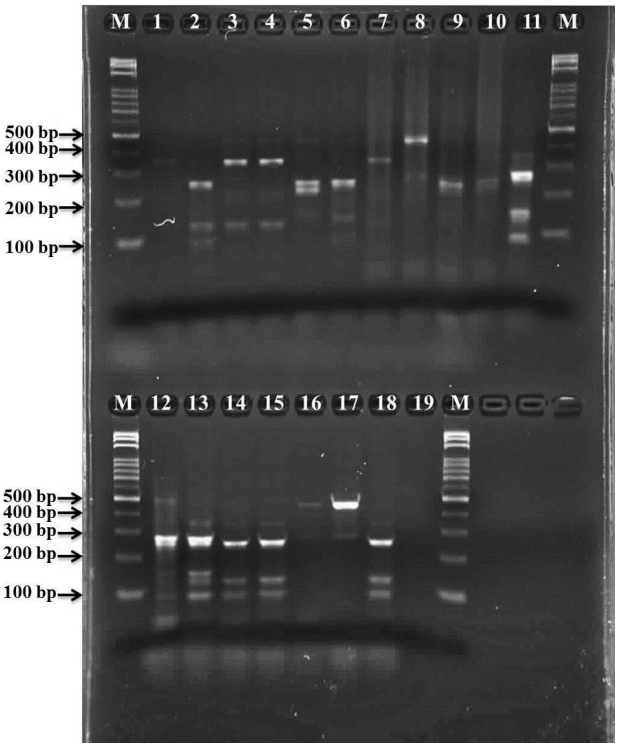

Fig. 5: Electrophoresis image of PCR-RFLP/*Bst*EII enzyme. Column M: 100 bp of DNA molecular weight marker (Amresco, 100 bp ladder, K180-250UL), Column 1-13: *Mycobacterium* spp. isolates, Column 14: *M. gordonae* (RSKK 14470), 15: *M. fortuitum* (ATCC 6841), 16: *M. chelonae* (RSKK 06064), 17: *M. aurum* (DSMZ 6695), 18: *M. tuberculosis* (H37Rv), 19: Negative control

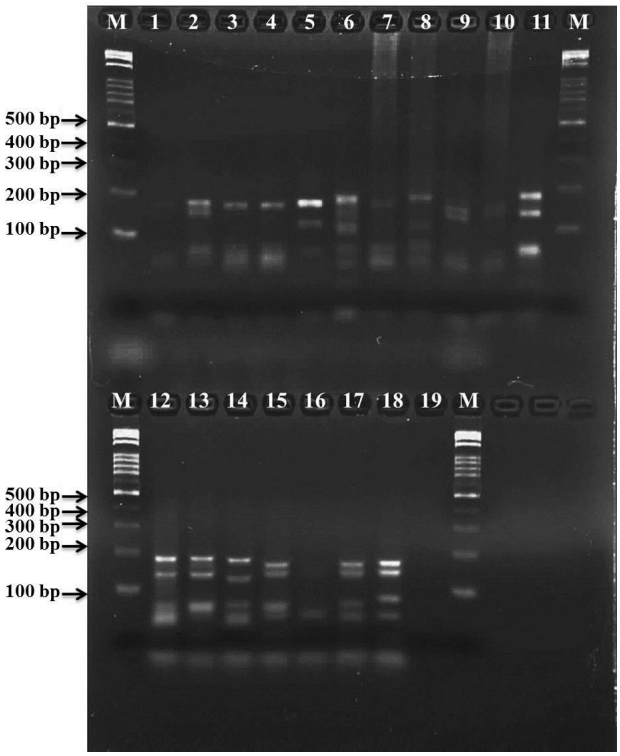

Fig. 6: Electrophoresis image of PCR-RFLP/*Hae*III enzyme. Column M: 100 bp of DNA molecular weight marker (Amresco, 100 bp ladder, K180-250UL), Column 1-13: *Mycobacterium* spp. isolates, Column 14: *M. gordonae* (RSKK 14470), 15: *M. fortuitum* (ATCC 6841), 16: *M. chelonae* (RSKK 06064), 17: *M. aurum* (DSMZ 6695), 18: *M. tuberculosis* (H37Rv), 19: Negative control

Table 2: *Mycobacterium* species according to season, fish tissues and isolates

Season	Fish Nr	Tissue	Isolates	*Mycobacterium* species
Autumn	13	Skin	A[a] 14 S[s]	*M. fortuitum*
Winter	28	Skin	A 28 S	*M. marinum*
Spring	46	Skin	A 46 S	*M. vaccae*
Winter	22	Internal organ	T[t] 22 I[i]	*M. aurum*
Spring	56	Skin	T 56 S	*M. scrofulaceum*
Spring	57	Skin	T 57 S	*M. scrofulaceum*
Autumn	68	Skin	T 68 S	*M. fortuitum*
Autumn	70	Skin	T 70 S	*M. scrofulaceum*
Autumn	72	Skin	T 72 S	*M. fortuitum*
Autumn	14	Skin	K[k] 14 S	*M. genavense*
Autumn	16	Skin	K 16 S	*M. genavense*
Autumn	18	Skin	K 18 S	*M. genavense*
Winter	42	Skin	K 42 S	*M. genavense*

[a] Anamur, [k] Karaduvar, [t] Taşucu, [S] Skin, and [i] Internal organs

mycobacteria in red mullet (*Mullus barbatus barbatus*) and surmullet (*Mullus surmuletus*) is documented for the first time. Furthermore, even though mycobacteriosis cases have been reported previously in farmed sea bass (Korun *et al*., 2005), this is the first study to isolate and identify *Mycobacterium* spp. in marine fish in Turkey.

Mycobacteria in wild marine fish have been reported at different rates. While *Mycobacterium* was detected in 25% of 20 silver mullet (*Mugil curema*) in Venezuela (Perez *et al*., 2001), 50% of wild rabbitfish (*Siganus rivulatus*) samples caught inside sea bass farming cages were infected by mycobacteria in Israel (Diamant *et al*., 2000). During a disease outbreak at Chesapeake Gulf in the United States, *Mycobacterium* was detected in 76% of 196 striped bass (*Moronesaxatilis*) samples (Rhodes *et al*., 2004). The ratio (6.25%) of mycobacteria in red mullet and surmullet in this study is relatively lower than those reported from other countries. Measures of *Mycobacterium* in wild fish were estimated to be larger in the vicinity of infected mariculture cages (Diamant *et al*., 2000). However, along the Mersin coastline, only three fish farms are located near Taşucu and no cases of mycobacterosis have been reported so far.

Many *Mycobacterium* species e.g. *M. chelonei* subsp. *abscessus*, *M. chelonei* subsp. *chelonei*, *M. chesapeaki*, *M. fortuitum*, *M. interjectum*, *M. marinum*, *M. montefiorense*, *M. pseudoshottsii*, *M. scrofulaceum*, *M. szulgai*, *M. shottsii*, and *M. triplex* have been reported in marine fish species to date (Lansdell *et al*., 1993; Diamant *et al*., 2000; Heckert *et al*., 2001; Perez *et al*., 2001; Levi *et al*., 2003; Whipps *et al*., 2003; Rhodes *et al*., 2004, 2005; Jacobs *et al*., 2009; Gauthier *et al*., 2010). This study is the first description of *M. fortuitum*, *M. marinum*, *M. vaccae*, *M. aurum*, *M. scrofulaceum*, and *M. genavense* in red mullet and surmullet in Turkey. As mentioned earlier, *M. fortuitum* was detected in many aquarium fish species (Marzouk *et al*., 2009), farmed silver mullet (*Mugil curema*) (Perez *et al*., 2001), and wild sea fish (Lansdell *et al*., 1993). In addition, *M. marinum* was isolated in aquarium fish (Pate *et al*., 2005; Marzouk *et al*., 2009), cultured yellowtail (*Seriola quinqueradiata*) (Weerakhun *et al*., 2007), rabbitfish (*Siganus rivulatus*) (Diamant *et al*., 2000) and turbot (*Scophthalmus maximus*) (Dos Santos *et al*., 2002). While *M. aurum* was reported in striped snakehead (*Channa striatus*) (Tortoli *et al*., 1996), *M. scrofulaceum* has been documented in wild silver mullet (*Mugil curema*) (Perez *et al*., 2001). The presence of *M. genavense* has been previously reported in water samples (Nichols *et al*., 2004). *M. vaccae*, regarded as environmental saprophyte mycobacteria, was used in the development of a vaccine against human tuberculosis (Yang *et al*., 2010). Previous literature reviews have not documented any cases of fish infections by *M. vaccae* and *M. genavense* yet. To our knowledge, this is the first detection of *M. vaccae* and *M. genavense* in fish.

Like other environmental mycobacteria, ichthyo-zoonotic mycobacteria are known to cause infections in humans with different degrees of severity, especially in immuncompromised individuals (Jacobs *et al*., 2009). *M. marinum*, *M. fortuitum*, and *M. scrofulaceum* are known to cause both fish and human diseases.. They have been mostly detected in cutaneous infections (Sanders *et al*., 1995; Rajadhyaksha *et al*., 2004) and also rarely in infections of the respiratory system, soft tissue and blood (Han *et al*., 2000). In recent years *Mycobacterium aurum* (Katalin and Ranalli, 2003) and *M. genavense* (Rammaert *et al*., 2011) have also been isolated as pathogens in immuncompromised patients.

It should be underlined that, although ichthyo-zoonotic mycobacteria have been isolated in red mullet and surmullet samples, they do not pose serious risks in terms of food safety, because these fish are cooked before consumption. Nevertheless, raw fish should be handled with care since injuries caused by fins of contaminated fish could pose severe health risks, especially for immuncompromised individuals (Decostere *et al*., 2004; Patel *et al*., 2007). Further biochemical and molecular analyses need to be carried out to understand the epidemiology and pathogenicity of the *Mycobacterium* spp. isolated in this study, both on fish and humans.

Acknowledgements

Authors would like to thank Dr. G. Emekdaş, Dr. G. Aslan and Dr. M. Ülger (Department of Microbiology, Faculty of Medicine, University of Mersin) for their support and contributions during the laboratory phase of this study, and S. Coban for editing the language of the manuscript.

This study was a Ph.D. dissertation of P. Sevim supported by the Scientific Research Unit of the University of Mersin, grant No. BAP-FBE YB (PB) 2009-3 DR.

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