First isolation of *Mycobacterium marinum* from sea bass (*Dicentrarchus labrax*) and gilthead sea bream (*Sparus auratus*) cultured in Turkey

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Abstract

*Mycobacterium marinum* is the most important fish tuberculosis agent. Turkey is an important Mediterranean country in terms of sea bass, sea bream and trout production. In this work, a total of 1050 fish samples (300 sea bass, 300 sea bream, 450 trout, respectively) taken randomly from 20 sea bass and sea bream (No:1-20), and 15 trout (No: 21-35) farms, were analysed using a combination of culture-based and molecular (Reverse Hybridisation) methods for *Mycobacterium* spp. The majority of samples were negative for mycobacteriosis. However, isolates that were positive by Reverse Hybridisation for *M. marinum* were isolated and identified from 60 fish (30 sea bass and 30 sea bream samples) from two cages of one farm in the Milas/Muğla province (Southeastern part of the Aegean Sea). This is the first *M. marinum* isolation from cultured sea bass and sea bream in Turkey.

Introduction

Aquaculture is an emerging sector within the Turkish economy. According to the data supplied by the The State of World Fisheries and Aquaculture (FAO, 2010), Turkey has shown the third highest rate of increase of aquaculture production, after China and India. In 2013, there were 2.353 aquaculture production premises, with 1935 freshwater and 418 marine sites. Sea bass and sea bream farms are predominantly located alongside the Mugla and Izmir coast of the Aegean Sea, especially in Milas. Other production sites are located in Trabzon, Ordu and Samsun provinces in the Black Sea Region; Hatay, Antalya and Mersin provinces in the Mediterranean Region and Çanakkale and Balıkesir in the Marmara Region. Trout farms are spread throughout Turkey (Anonymous, 2015). Infectious disease is an important issue

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which hinders the fast growth rate of the aquaculture sector (Aiello and Mays, 2006).

*Mycobacterium marinum* is a recognized fish pathogen (Austin and Austin, 1987; Colorni, 1992), that can also infect mammals, including humans (Smith and Willett, 1980). Initially isolated from sea bass (*Dicentrarchus labrax*) cultured in the Red Sea in 1990 (Colorni, 1992), the agent has since been reported from more than 20 fish species (Frerichs, 1993). It has also recently been recovered from a Turkish species, meagre (*Argyrosomus regius*) (Avsever et al., 2014).

*Mycobacterium marinum* may cause local (Knibb et al., 1993) or systemic (Frerichs, 1993) infections in fish. Skin lesions are predominant in local infections, whereas in systemic infections stunted growth and tubercule formation is commonplace, especially in internal organs such as the spleen and kidneys (Avsever et al., 2014). Secondary infections and an increase in mortality may be seen in both cases. Even when death is averted; weight loss and deformed appearance lower the market value of fish. Antibiotic treatment is not considered to be economic or practical. The main methods of disease control and management include marketing the fish as fillets, following harvest, disinfecting the cages after depopulation and fallowing for a year. *Mycobacterium* spp., can maintain their viability in the organic debris layer on the cages (Colorni, 1992; Frerichs, 1993; Ucko and Colorni, 2005; Wayne and Kubica, 1986).

*Mycobacterium marinum* is a threat to human health. The agent can cause granulomas on the surface of hands and in tendons following contact with infected fish or contaminated water (Williams and Riordan, 1973; Ucko and Colorni, 2005). As symptoms and blood tests are mostly non-specific, diagnosis is generally delayed and this leads to a series of complications such as tendon rupture, osteomyelitis, loss of motion and even amputation (Barton et al., 1997; Lee et al., 2004).

There are no previous reports on *M. marinum* isolation from sea bass and sea bream cultured in Turkey. Although Avsever et al. (2014) isolated *M. marinum* from meagre cultured in the Milas region of Turkey.

The main method for *M. marinum* diagnosis is isolation (Koneman et al., 1992) and identification is mostly carried out with molecular methods. Polymerase Chain Reaction (PCR) and Restriction Fragment Length Polymorphism (RFLP) are used successfully for *M. marinum* identification (Smith and Willett, 1980; Talaat et al., 1997). However, Reverse Hybridisation (RH) is a rapid and accurate identification method (Posteraro et al., 1998; Sanguinetti et al., 1998).

We report the first isolation of *M. marinum* from cultured sea bass and sea bream in Turkey.

**Materials and methods**

Thirty five fish farms were chosen from a list of premises supplied by the General Directorate of Fisheries and Aquaculture, Ministry of Food, Agriculture and Livestock. A total of 1050 harvest size (200-400g) cultured fish (300 sea bass, 300 sea bream, 450 trout samples) were sampled at random from farms (Table 1). Also, 40 wild fish samples (14 grey mullet, 12 sea bass, 4 sea bream) taken from the proximity of the cages that contained *M. marinum* positive fish were also included in the study. All the samples were taken in the summer (June and July).
Table 1. Sample information and study results.

<table>
<thead>
<tr>
<th>Provinces</th>
<th>Number of farms/Number of fish</th>
<th>Region</th>
<th>Number of fish Positive for <em>M. marinum</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Muğla (Milas)</td>
<td>12 (Farm no: 1-12) /360*</td>
<td>Southern Aegean</td>
<td>60 (30 sea bass, 30 sea bream; in two cages of one farm- Farm no:1-)</td>
</tr>
<tr>
<td>İzmir</td>
<td>4 (Farm no: 12-16/120*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aydın</td>
<td>1 (Farm no: 17)/30*</td>
<td>Northern Aegean</td>
<td>-</td>
</tr>
<tr>
<td>Çanakkale</td>
<td>1 (Farm no: 18) /30*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trabzon</td>
<td>1 (Farm no: 19) /30*</td>
<td>Black Sea</td>
<td>-</td>
</tr>
<tr>
<td>Mersin</td>
<td>1 (Farm no: 20) /30*</td>
<td>Mediterranean</td>
<td>-</td>
</tr>
<tr>
<td><strong>Total sea bass/bream: 600</strong>*</td>
<td></td>
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</tbody>
</table>

Sea bass/bream farms and number of fish samples

<table>
<thead>
<tr>
<th>Provinces</th>
<th>Number of farms/Number of fish</th>
<th>Region</th>
<th>Number of fish Positive for <em>M. marinum</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Trabzon</td>
<td>1 (Farm no: 21) /30</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Artvin</td>
<td>1 (Farm no: 22) /30</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rize</td>
<td>2 (Farm no: 23-24) /60</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ordu</td>
<td>1 (Farm no: 25) /30</td>
<td>Black Sea</td>
<td>-</td>
</tr>
<tr>
<td>Tokat</td>
<td>1 (Farm no: 26) /30</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gümüşhane</td>
<td>1 (Farm no: 27) /30</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sivas</td>
<td>1 (Farm no: 28) /30</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mersin</td>
<td>1 (Farm no: 29) /30</td>
<td>Mediterranean</td>
<td>-</td>
</tr>
<tr>
<td>İzmir</td>
<td>6 (Farm no: 30-35) /180</td>
<td>Aegean</td>
<td>-</td>
</tr>
<tr>
<td><strong>Total trout: 450</strong></td>
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</tbody>
</table>

* Half sea bass, half sea bream
water temperatures during the sampling were as follows: Aegean Sea (25 ±2°C), Mediterranean (28 ±2°C), Black Sea (23 ±2°C), Marmara sea (24 ±2°C), respectively. Freshwater temperatures during trout sampling were 15 °C (±4 °C).

**Isolation of Mycobacterium spp.**

Internal organ samples (spleen, liver, kidney) of fish were decontaminated first with hexadecylpyridinium chloride (HPC). For this, the pellets formed after centrifugation were re-suspended in 10 mL 2% (w/v) HPC (Sigma) and incubated for 5 h at room temperature (21°C). Following incubation, the samples were centrifuged (2500 x g, 15 min) and the pellets resuspended in 1 mL PBS. A slide was prepared from this PBS solution, stained using Ziehl-Neelsen method and examined for the existence of acid-resistant bacilli. For primary isolation inoculations were made on Löwenstein-Jensen (L-J) media and BACTEC MGIT 960. Incubated media were incubated for 28 days at 30°C (Avsever et al., 2014; Koneman, 1992; Posteraro et al., 1998; Sanguinetti et al., 1998).

**Identification with Reverse-Hybridisation method**

Colonies taken from the L-J medium were identified with a Reverse Hybridisation based GenoType Mycobacterium assay CM/AS (Hain Lifescience/ Germany). For this purpose, hybridisation of biotin conjugated amplicons from PCR with species-specific oligonucleotides were carried out on nitrocellulose bands. DNA/DNA hybrids were visualised with enzymes and chromogenic substrates. Resulting patterns were evaluated according to the data supplied by the manufacturer (Avsever et al., 2014; Posteraro et al., 1998; Sanguinetti et al., 1998).

**Results and discussion**

Mycobacteriosis was not detected in any of the fish collected from 34 of the 35 farm samples. However, it was detected at apparent high frequency from 30 sea bass and 30 sea bream samples from two cages of one farm (100%) in Milas. In these fish, skin lesions with varying severity (Figure 1), cachexia and pale coloring were observed. In necropsy, tubercules were observed in the internal organs (Figure 2, 3). Inoculations from these fish resulted in bacterial colony formation on L-J media by three weeks post-inoculation. These samples were positive in the BACTEC MGIT 960 system. Isolates were identified using Reverse Hybridisation and all gave the patterns 1, 2, 3, 10, 15 which are specific to *M. marinum* and *M. ulcerans* (Figure 4). As isolates were photochromogenic (Figure 5), they were concluded to likely be *M. marinum* (*M. ulcerans* is not photochromogenic). Sampling data and study results are supplied in Table 1.

Clinical findings of piscine mycobacteriosis vary with the host and environmenta! conditions; however, lethargy, inappetence and exophthalmia are typical general symptoms (Avsever et al., 2014). In this study, 60 of the fish examined and found to be positive for *M. marinum* had skin lesions of varying severity, paleness, cachexia and tubercule formation in internal organs. These findings are paralleled to fish tuberculosis symptoms (Austin and Austin, 1987).

Detection of *M. marinum* in the sea bass/sea bream in the Milas region was completed after a previous study by Avsever et al. (2014) regarding the isolation of *M. marinum* from meagre in the same region. There were about two km between the farms containing *M. marinum* posi-
Figure 1. Severe skin lesion from a *M. marinum* positive sea bass.

Figure 2. Enlargement of spleen and tubercule formation from a sea bass with *M. marinum*.

Figure 3. Enlargement of spleen and tubercule formation from a sea bream positive for *M. marinum*. 
tive meagre samples (Avsever et al., 2014) to those with *M. marinum* positive Sea bass and Sea bream samples (in this work). The short period of time between these two reports is an indication of the spread of the disease. In contrast, the bacterium was not isolated from 300 fish samples from the Milas region (Southern Aegean) or 180 samples from the Northern part of the Aegean Sea. This might be an indication that the disease is limited to a part of the Milas region. However, as the Milas region is an important center for mariculture, there are a high number of stocks at risk. In this study, no *M. marinum* isolates were obtained from sea bass or sea bream samples taken from the Mediterranean or Black Sea regions and trout samples taken from all of Turkey.

The ‘gold standard’ method for *M. marinum* diagnosis is isolation. Molecular methods are generally employed for the identification and confirmation of the agent. In this work, reverse

Figure 4. Reverse Hybridisation test, 1, 2, 3, 10, 15 patterns specific to *M. marinum* and *M. ulcerans*.

Figure 5. *M. marinum* colony grown on L-J medium (Sea bass, primary isolation). Photochromogenic properties present in *M. marinum* and absent in *M. ulcerans*.
hybridisation, which is a rapid and accurate method, was used for identification (Avsever et al., 2014; Posteraro et al., 1998; Sanguinetti et al., 1998).

For treatment of *M. marinum* infections, kanamycin, streptomycin, isoniazid and rifampin combination, doxycycline or minocycline with sulphasoxazole can be used. However, treatment is expensive and time consuming. Also, it is not possible to eradicate *M. marinum* from a population with antibiotic therapy. Still, treatment might be an option for valuable broodstocks (Aiello and Mays, 2006; Andrews et al., 1988). Filleting fish, following a harvest of the stocks, disinfecting the cages and leaving them fallow for a year are recommended methods for disease control. *Mycobacterium* spp., can remain viable in the organic debris layer on the cages even after disinfection. For this reason, it is necessary to clean this layer.

Avsever et al. (2014) previously did not isolate microbacteriosis agents from wild fish caught from outside the *M. marinum* positive meagre cages and stated that population density was an important cause of morbidity for this disease. In this work, wild fish were caught from outside the cages of *M. marinum* positive fish and again no *M. marinum* was isolated in them.

As a result of this work, *M. marinum* was isolated for the first time from sea bass and sea bream stocks in Turkey; zoonotic characteristics of the disease were emphasised and protection/control measures for the disease were discussed.

References


