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Research article

MYCOBACTERIOSIS IN FARMED SEA BREAM (*Sparus aurata*) CAUSED BY *Mycobacterium frederiksbergense* IN TURKEY

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Over the past decades, different mycobacteriosis agents have become important fish pathogens. The present study describes a different mycobacteriosis in farmed sea bream (*Sparus aurata*) caused by *Mycobacterium frederiksbergense* in Turkey. Affected 15 fish, weighing 15 to 20 g, showed lethargy, stunted growth, pale skin, dorsal fin necrosis, and a significant level of mortality (40 %) in fish stocks. Internally, no multifocal white-colored granulomas in the visceral organs were observed. Inoculation of the visceral organs onto Löwenstein-Jensen medium and Tryptic Soy Agar (1.5% NaCl) slants produced only fast-growing (2-3 weeks), orange to yellow-colored, photochromogenic acid-fast colonies. Ziehl-Nielsen positive bacterial isolates were identified using a commercially available line probe assay (Genotype Mycobacterium CM/AS assay) and with 16S rRNA gene sequencing analysis based on 16S rRNA gene sequencing, fifteen isolates were identified as *Mycobacterium frederiksbergense*. Histopathologically, epithelioid cell granulomas were not observed in any vicseral organs but acid-fast bacteria were detected in the liver, kidney, spleen, and heart tissue. This study shows that asystemic mycobacteriosis is observed in sea bream with high mortality.

Key words: *Mycobacterium frederiksbergense*, sea bream, Genotype Mycobacterium CM/AS assay, 16S rRNA gene

INTRODUCTION

Occurrence of systemic fish mycobacteriosis, causing high mortality, has increasingly been reported in the past decade [1-5]. This is a systemic disease among both wild and cultured fish species [6,3]. The causative agents of mycobacteriosis most commonly reported in fish include *M. marinum*, *M. fortuitum* and *M. chelonae*, though other species including *M. shottsii* [7], *M. pseudoshottsii* [8], *M. gordonae* [9], *M. montefiorense* [10], *M. stomatepiae* [11], and *M. haemophilum* [12] have also been reported. This *Mycobacterium*

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species affects a variety of fish such as Atlantic salmonids [13], rabbit fish [14], sea bass [15,16], sea bream [2,17], turbot [18], cultured striped bass [19], meagre [1,5], and striped bass [20].

Nontuberculous mycobacteria (NTM), which are slow or rapidly growing, have been rarely observed as opportunistic infections [21,22]. *Mycobacterium frederiksbergense,* short rods or coccoid acid-fast bacteria, [23] are environmentally important, for example in bioremediation reactions [24] and also have been isolated and identified from alfalfa plants [22] and striped bass (*Morone saxatilis*) [25].

Economically, the sea bream is the most important marine fish species cultured in Turkey. A significant mortality (40%) occurred in diseased sea bream cultured in the Aegean Sea. Bacteriologic, molecular, and histopatologic analyses demostrated that *Mycobacterium frederiksbergense* caused significant mortality in the moribund sea bream. This paper is the first report of mycobacteriosis caused by *M. frederiksbergense* in cultured sea bream.

MATERIAL AND METHODS

Fish Samples

Fifteen affected fish (15-20 g) that generally showed loss of appetite, lethargy, and emaciation were obtained from a floating marine cage farm located on the coast of the Aegean Sea in Turkey.

Bacteriology

Samples of kidney, liver, and spleen were streaked onto Tryptic Soy Agar (TSA) supplemented with 1.5% NaCl and Löwenstein-Jensen (L-J) medium. Plates were incubated at 24-25 C° for 2 weeks. Physiologic characteristics were determined using some biochemical tests such as catalase, nitrate reduction, urease activity, hydrolyse Tween 80, and growth on MacConkey Agar [26].

Molecular Studies

The isolated acid-fast bacteria (n=15) were identified using commercially available line probe assays, the Genotype CM and AS (HainLife Science, Germany). Isolates on L-J medium were prepared by suspending a loopful of the bacteria in 1 ml of distilled water. DNA extraction was performed through sonication for 15 min, followed by heating to 100 C° for 15 min. Samples were then centrifuged for 2 min and the supernatant was used for the assay. The GenoType Mycobacterium CM/AS assay was performed in accordance with the manufacturer's instructions. Identification of the isolate was performed by sequencing the 16S rRNA gene according to the classic method of Kirschner et al. [27].

Histopathology

Tissue samples from the kidney, liver, spleen, heart, gut, brain, and gills were fixed in 10% buffered formalin and processed for paraffin embedding. Histologic sections (4-5 μm) were stained with hematoxylin-eosin (HE) and Ziehl-Nielsen (ZN) and examined under light microscopy [28]. In addition, the same protocol was applied for the two healthy sea breams as the control in the histopathological investigation.

RESULTS

Clinical Findings

Fifteen moribund sea breams exhibited external clinical signs that included extreme emaciation (*) pale skin, loss of scales, and dorsal and pectoral fins necrosis (Figure 1a), saddleback lesions (Figure 1b). Internally, the moribund fish showed a pale liver, enlargement of the spleen, and yellow gelatinous fluid in the intestine (Figure 2b).

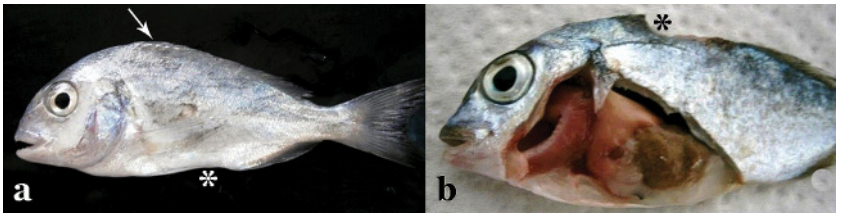


Figure 1. Emaciation (*), pale skin, loss of scales, dorsal fin necrosis (arrowed) **(a)**; saddleback lesion (*), pale liver and gill necrosis **(b)**

Bacteriological Findings

After the two-week incubation period, only yellow to orange pigmented colonies were observed both on L-J medium (Figure 2a) and also on TSA (Figure 2b). Smooth, photochromogenic colonies were examined for acid-fastness and Gram staining. The ZN stained bacterial smear from these colonies revealed acid-fast and short rod or coccoid-shaped bacteria (Figure 2c). There was also a positive reaction for catalase and nitrate reduction; the test for urease was negative and there was no growth on MacConkey Agar (MCA).

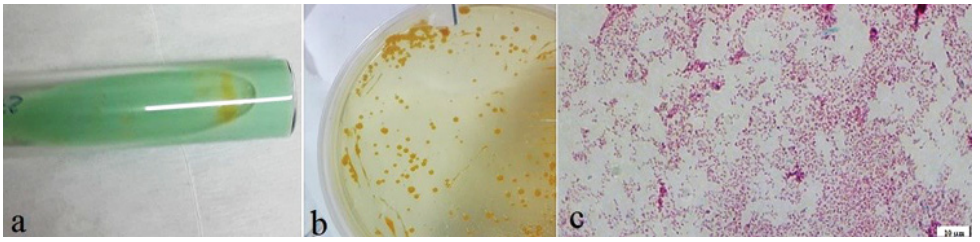


Figure 2. Yellow-orange pigmented colonies on Lowenstein-Jensen slants **(a)**; on TSA **(b)**; acid-fast bacterium from L-J slant **(c)**

Molecular Studies Findings

The Genotype CM yielded bands on position 1, 2, 3, and 10 (Figure 3a), resulting in *Mycobacterium* species, and Genotype AS test bands were detected at position 1, 2, 3, and 12 (Figure 3b), again yielding *Mycobacterium* species. According to these results, all mycobacterial isolates were not identified to the species level with Genotype AS and CM kits.

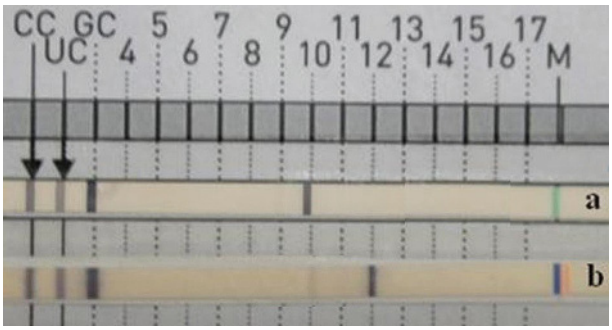


Figure 3. The results of GenoType Mycobacterium, CM (1,2,3, and10th band) **(a)**; AS (1,2,3, and 12th band) **(b)**

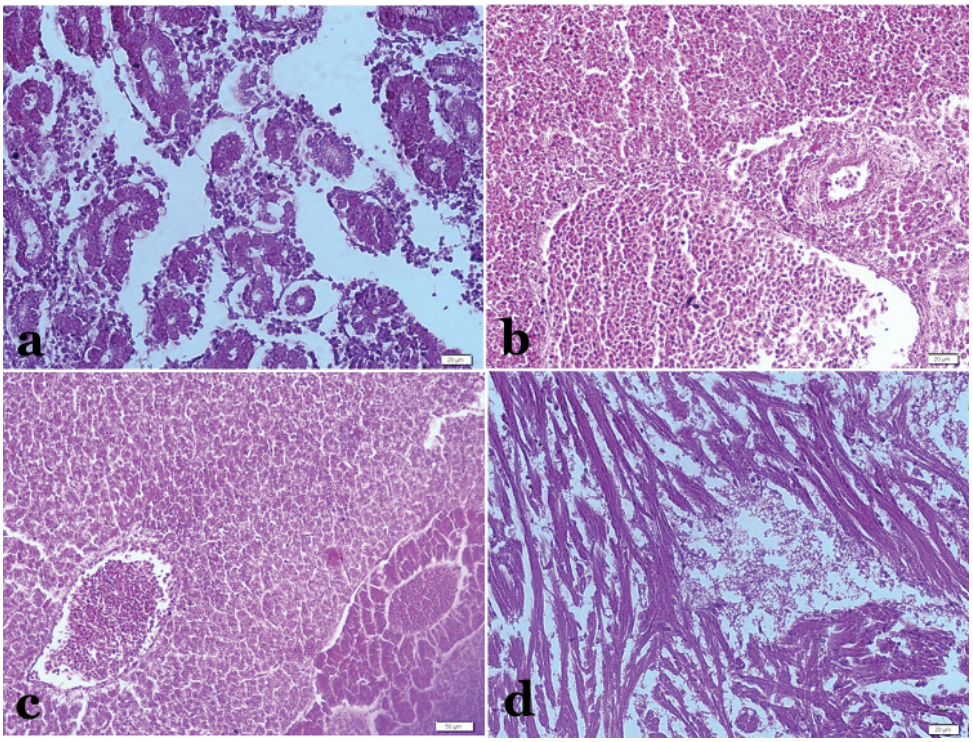


Figure 4. Histopathological section of the kidney **(a)**, spleen **(b)**, liver **(c)**, and heart mucle **(d)** stanining with HE

Gene sequencing with 16S rRNA revealed that the fifteen isolates were *M. frederiksbergense*. This result was derived from the National Center for Biotechnology Information (NCBI) blast database (accession number LN613126; Benedek et al.). The sequence obtained in this study is defined as GenBank accession number MF431727.

Histopathological Findings

The epithelioid cell granulomas were not observed in any visceral organs (4a,b,c,d), but acid-fast bacteria were observed in the kidney (Figure 5a), spleen (5b), liver (5c), and heart tissue (5d).

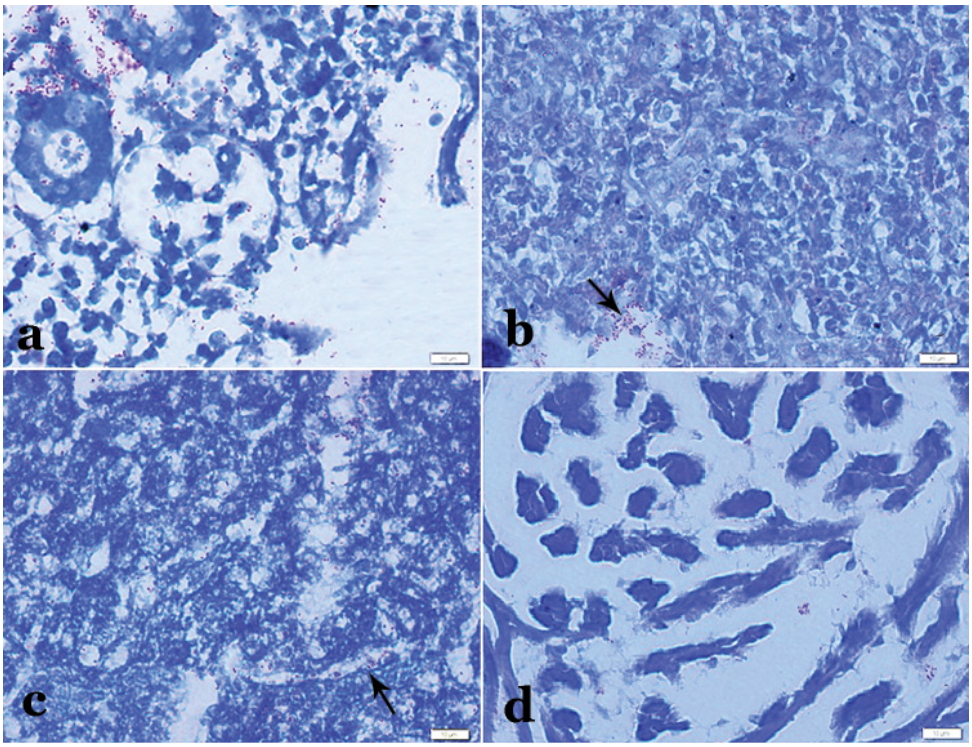


Figure 5. Pink colored, acid-fast bacteria in the kidney **(a)**, spleen **(b)**, liver **(c)** and heart tissue **(d)** section stanining with ZN (arrowed)

DISCUSSION

Since the first description of mycobacteriosis in carp in 1897, mycobacteriosis has become the most common chronic disease of fish. Although the causative agents of mycobacteriosis such as *M. marinum*, *M. fortuitum* and *M. chelonae* have been commonly reported from a wide range of diseased fish in the recent years, a new species of mycobacteria related to NTM such as *M. shottsii* [7], *M. pseudoshottsii* [8], *M. gordonae* [9], *M. montefiorense* [10], *M. stomatepiae* [11], and *M. haemophilum* [12] have been reported

in both cultured and wild fish species. In Turkey, only one *Mycobacterium* species, *M. marinum*, has been reported from diseased sea bass [16], meagre [1, 5], as well as sea bream [2]. There were no reports about any other mycobacteriosis agents isolated from diseased cultured and wild fish species.

The clinical signs of mycobacteriosis in fish have been reported to be unspecific [3]. In the present study, the clinical findings were similar to those previously described by Colorni et al. [29], Rhodes et al. [30], and Swanson et al. [31]. However, confusingly, external saddleback lessions were observed, different from the ones described in these publications.

For the presumptive diagnosis of mycobacteriosis, macroscopic grayish-white miliary granulomas were not observed on the visceral organs as described in previous reports [3,32,33]. For this reason, our diagnosis was solely based on the culture of causative microorganisms from the kidney, spleen, and liver of affected sea bream using selective L-J and TSA medium. However, phenotypic characteristics such as photochromogenic yellow to orange-pigmented colonies, a shorter incubation period (2-3 weeks), and conventional methods such as catalase, nitrate reduction, urease were not sufficient for the identification of the isolates. Also, colony formation and color of the isolates were very similar to those of *M. marinum*, as described in previous reports [3,5,18,20,34]. The most important differentation between our isolates and *M. marinum* was the incubation period because the isolates in the present study had a shorter incubation period compared with the incubation period of *M. marinum.*

Our fifteen isolates were Gram-positive, non-spore forming, acid-fast and non-motile. They were short rod-shaped or coccid. The isolates produced catalase and were positive for nitrate reductase but negative for urease. It was able to hydrolyse Tween 80 but did not grow on MacConkey agar. These results are similar to the biochemical results of *M. frederiksbergense* [24] but these biocehmical results were not sufficient for the identification of the acid-fast bacteria.

In this study, no epithelioid cell granulomas were observed in any viseral organs but acid-fast bacteria were demostrated in the liver, kidney, spleen, and heart tissue by ZN stain. The primary pathologic lesion associated with mycobacteriosis is classic granulomatous inflammation [3,33] yet it has been reported that *M. shottsii* and *M. gordonae* rarely produce epithelioid cell granulomas [35] as in this study. Also, Yanong et al., [36] reported severe systemic mycobacteriosis without typical granuloma formation in the moribound frogfish (*Antennarius striatus*).

Mycobacterium species have been identified to the species level using Genotype Mycobacterium AS and CM assays in previous reports [1,37-40]. However, Timur et al. [5] reported that *M. marinum* species isolated from meagre were identified as *M. ulcerans* according to AS assay. In the present study, these molecular assays were insufficient to identify our isolates, because our isolates were only identifed as *Mycobacterium* species according to both AS assay and CM assay. Consequently, we used another molecular technique to identify our isolates. Sequencing of 16S rRNA

has been used for the differentiation and classification of *Mycobacterium* species [24], so our isolates were identified using this method. According to this molecular method, isolates were identified as *M. frederiksbergense*. The sequence obtained in this study is defined as GenBank accession number MF431727.

M. frederiksbergense species are phylogenetically closely related to *Mycobacterium diernhoferi, Mycobacterium neoaurum*, and *Mycobacterium hodleri*. Members of this group of mycobacteria are able to degrade polycyclic hydrocarbons [24] and are thus considered to be highly adapted to environmental niches. But they are capable of fish infection as described in previous reports [25]. Also Talaat et al. [41] reported that *M. smegmatis,* non-pathogenic mycobacterium species, has been shown to be pathogenic in fish. These result indicates that enviromental isolates such as *M. frederiksbergense* may play a role in mycobacterial disease of fish. In conclusion, this study represents the first report of *M. frederiksbergense* isolation and identification from moribund sea bream in the Aegean Sea.

Authors' contributions:

UC conceived of the this study, did fish sampling and carried out histopathology, substantial contributions to conception and design, or acquisition of data, and interpretation of data. GEG, FW, EZ and PG carried out the molecular genetic studies. GEG and EZ carried out the Genotype Mycobacterium CM/AS assay. FW and PG participated in the 16 S rRNA sequence analysis.

Declaration of conflicting interests:

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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MIKOBAKTERIOZA KOD ORADE IZ UZGOJA (*SPARUS AURATA*) IZAZVANA SA *MYCOBACTERIUM FREDERIKSBERGENESE* U TURSKOJ

URKU Cigdem, GENC Gonca Erkose, WITTWER Franziska, ERTURAN Zayre, PFYFFER Gaby

U proteklih nekoliko dekada, različite vrste mikobakterija su postale značajni patogeni riba. Studija opisuje različite mikobakterioze kod orada u uzgoju (*Sparus aurata*), izazvane sa *Mycobacterium frederiksbergenese* u Turskoj. Petnaest inficiranih riba, težine 15 do 20 grama, pokazivale su znakove letargije, zaostajanja u rastu, bledilo kože, nekroze dorzalnog peraja kao i značajan nivo mortaliteta (40%) u ukupnoj populaciji riba. Prilikom obdukcije, nisu uočene multifokalne belo obojene granulomatozne promene u visceralnim organima. Inokulacijom uzoraka iz visceralnih organa u Löwenstein-Jensen hranljivi medijum kao i Tryptic soja agar (1,5% NaCl) kosi agar, dovelo je do brzog rasta (u roku od 2 do 3 nedelje), narandžasto do žuto obojenih, fotohromogenih acidorezistentnih kolonija. Izolovane bakterije su mogle da se oboje specijalnom metodom bojenja tj. bile su Ziehl-Nielsen pozitivne. Korišćenjem komercijanih dijagnostičkih kitova (Genotype *Mycobacterium* CM/AS metoda) uz sekvencioniranje 16S rRNK na osnovu sekvencioniranja 16S rRNK gena, identifikovani su izolati *Mycobacterium frederiksbergenese*. Histopatološki, granulomi nisu uočeni ni u jednom visceralnom organu, međutim acidorezistentne bakterije su dokazane u tkivu jetre, bubrega, slezine i srčanom mišiću. Ova studija ukazuje na asimptomatske mikobakterioze kod orade koje su praćene visokim stepenom mortaliteta.