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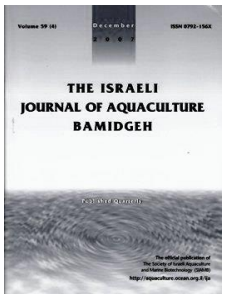
ISSN 0792 - 156X

PUBLISHER:

Israeli Journal of Aquaculture - BAMIGDEH - Kibbutz Ein Hamifratz, Mobile Post 25210, ISRAEL

Phone: + 972 52 3965809

http://siamb.org.il



Systemic Mycobacteriosis Caused by *Mycobacterium marinum* in Farmed Meagre (*Argyrosomus regius*), in Turkey

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(Received 26.9.2014, Accepted 15.1.2015)

Key words: *Mycobacterium marinum*, meagre, granulomatous lesions

Abstract

This paper describes systemic mycobacteriosis caused by *Mycobacterium marinum,* in farmed meager (*Argyrosomus regius*), in Turkey. Infected two year old fish showed signs of stunted growth, emaciation, slight ascites and exophtalmia, pale gills and significant mortalities. Only one fish sample showed hemorrhagic ulcerative skin lesions at the base of the caudal fin. Internal multifocal white colored granulomas in the spleen, kidney, and liver were observed. Ziehl-Neelsen (ZN) and Gram stained fresh squash mounts of the granulomas revealed Gram and ZN positive rods. Inoculation of sterile homogenates of the visceral organ granulomas on Lowenstein-Jensen slants produced slow-growing (3-4 weeks), yellow to orange colored, photochromogenic acid fast colonies. ZN positive bacterial isolates were identified using commercially available line probe assays (Genotype Mycobacterium CM/AS assay) and *hsp65* gene sequencing analyses. According to molecular analysis results, the isolates were identified as *Mycobacterium marinum*. Epithelioid cell granulomas were microscopically observed in the visceral organs and gills. ZN stained tissue sections exhibited heavy acid-fast rods within the granulomas.

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Introduction

Mycobacteriosis, caused by several species of the genus *Mycobaterium,* has been described as a systemic, serious, lethal, chronic, progressive, bacterial disease affecting wild and cultured marine, brackish, and freshwater fish worldwide. Although *Mycobacterium marinum* is considered to be the primary causative agent of fish mycobacteriosis, a number of *Mycobacterium* species such as *M. marinum*, *M. fortuitium, M. chelonae, M. salmoniphilum, M. smegmatis, M. abscessus, M. neonarum, M. simiae* and *M. poriferae* have been identified and are associated with tubercule granulomas in cultured, aquarium, and wild fish populations (Frerichs, 1993; Chinabut, 1999; Puttinaowarat et al., 2000; dos Santos et al., 2002; Toranzo et al., 2005; Pourahmad et al., 2009; Jacobs et al., 2009; Gauthier and Rhodes, 2009; Novotny et al., 2010).

Mycobacteriosis was reported in Pacific and Atlantic salmonids (Arakawa and Frayer 1984), rabbit fish (Diamant et al., 2000), sea bass (Colorni, 1992; 1996), sea bream (Colorni et al., 1996), farmed turbot (dos Santos et al., 2002), cultured striped bass (Hedrick et al., 1987) and in striped bass (Rhodes et al., 2004). Multiple granulomas were scattered or grouped in the visceral organs of various fresh water and salt water fish species (dos Santos et al., 2002; Rhodes et al., 2004; Toranzo et al., 2005; Gauthier et al., 2009; Jacobs et al., 2009).

Farming meagre in floating marine cages on the Aegean Sea coast began in the early 2000's. In September 2013, a large number of deaths occurred in 2 year old fish in a marine cage farm. Clinical, bacteriological, histopathological, and molecular examinations indicated that mycobacteriosis was present in the diseased meagre. This paper describes systemic mycobacteriosis in cultured meagre in Turkey.

Materials and Methods

Fish: Six fish (350-400g) showing signs of loss of appetite, lethargy, emaciation, and floating on the surface of the water were obtained from a floating marine cage farm located on the coast of the Aegean Sea in Turkey.

Bacteriology: Non-fixed tissue samples from liver, spleen, and kidney, including white colored granulomas were frozen by immersion in liquid nitrogen at -196 °C. These tissue samples were removed from the liquid nitrogen and brought to room temperature in the microbiology laboratory for the preparation of Gram and Ziehl-Neelsen (ZN) stained smears. The stained smears were examined by light microscopy.

To obtain a pure culture of mycobacteria, decontaminated homogenates were prepared from the visceral organ granulomas of the infected fish samples. The visceral granulomas were homogenized using sterile pestles and treated with 4% NaOH (w/v) at room temperature for 15 minutes and centrifuged at 8000 *g* for 15 minutes. The supernatant was removed, the pellet was washed twice with 1ml of sterile phosphate-buffered solution (PBS) and centrifuged one more time as described above. The pellet was re-suspended in 150 µl sterile PBS and twenty-five µl of this suspension was inoculated onto Lowenstein-Jensen medium incubated at 24-25°C (Pourahmad et al., 2009).

Molecular Study: The isolated ZN positive bacteria were identified using commercially available line probe assays of the Genotype CM and AS (HainLife Science, Germany). For preparing DNA, one loop of cells was suspended in 300 µL distilled water, boiled at 95C° for 20 min, sonicated for 15 min, and centrifuged for 5 min. The GenoType protocol consists of PCR amplification, hybridization of the PCR products to the probe-containing test strips, and detection of bound products (Richter et al., 2006). Sequencing of the 65-kDa heat shock protein gene (*hsp65)* was also performed (Pourahmad et al., 2009).

Histology: The kidney, liver, spleen, heart, and gill tissues with colored granulomas were processed for histopathology by fixing in 10% buffered formalin, and processed for paraffin embedding. Histological sections (4-5µm) were stained using hemotoxylin and eosin (H&E) and tissue ZN staining methods, and then examined by light microscopy (Bullock, 1978).

Results

Clinical Signs: Six, 2 year old, affected fish (weight 350-400g) exhibited nonspecific external clinical signs which included emaciation, stunted growth, and ascites (Fig. 1), slight exophtalmia (Fig. 2a), pale gills, and mortality. Among the six affected fish only one of them showed hemorrhagic ulcerative skin lesions at the base of the caudal fin (Fig. 2b). Gross internal signs of the affected fish included slight hemorrhagic ascites and characteristic white multifocal granulomas measuring 2-7 mm in the spleen, liver and kidney (Fig. 3a, b, c, d).

Fig. 1. Affected fish showed emaciation and stunted growth

Fig. 2. (a) Slight exophtalmia, (b) Hemorrhagic ulcerative skin lesions at the base of caudal fin

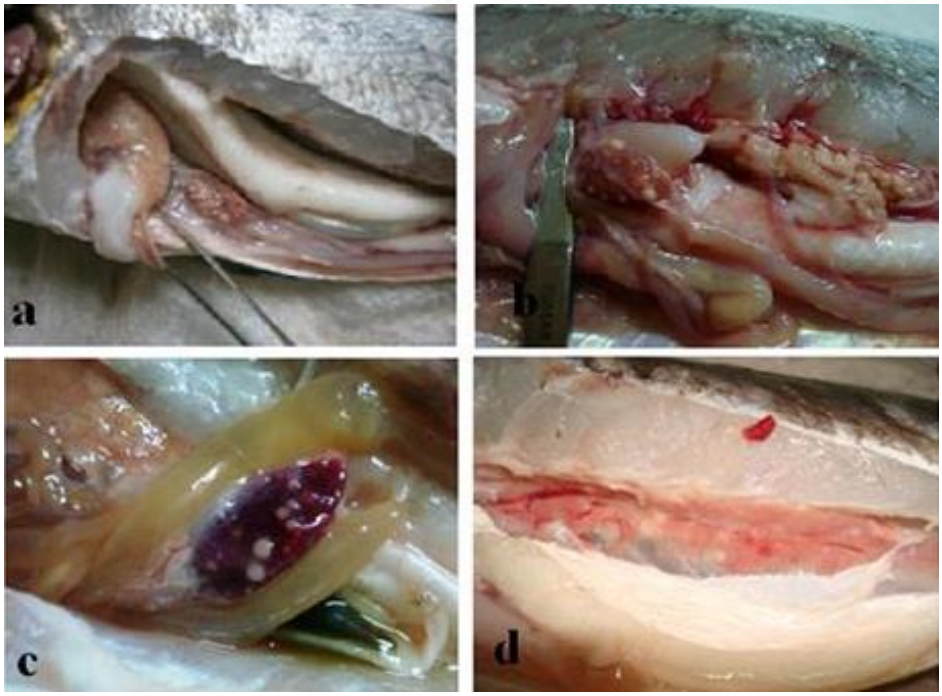

Fig. 3. Multifocal white granulomas (a) in the liver and spleen, (b) in the spleen and kidney, (c) in the spleen, (d) in the kidney

Bacteriology: ZN and Gram stained fresh squash mounts revealed ZN and Gram positive rods within the affected visceral organ nodules. After 3-4 weeks, sterile

homogenate inoculations on Lowenstein-Jensen slants produced slow growing yellow-orange pigmented colonies recovered from the visceral organ (kidney, spleen and liver) granulomas (Fig. 4a). Colonies were examined for acid-fastness. The ZN stained bacterial smears from these colonies revealed acid fast cross barring shaped rods (Fig. 4b).

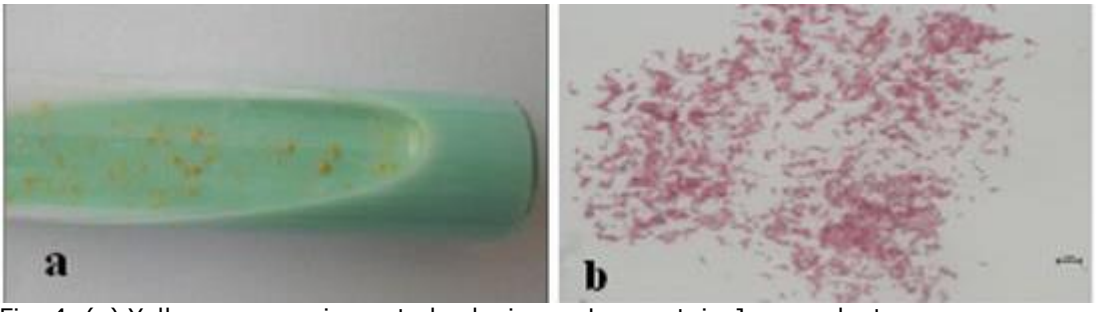

Fig. 4. (a) Yellow-orange pigmented colonies on Lowenstein-Jensen slants,
(b) Acid fast rods from Lowenstein-Jensen slants

Genotype Assay: The ZN positive isolates produced 10th and 15th bands on the Genotip CM (line a) (Fig. 5a). According to the interpretation chart, these isolates were determined to be *M. ulcerans* or *M. marinum*. Later these two species were further differentiated with a Genotype AS kit. According to the interpretation chart, these isolates were identified as *M. ulcerans* (Fig. 5 b).

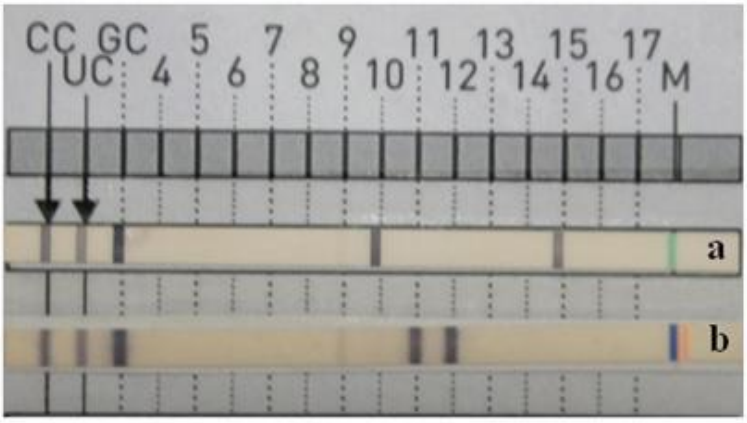

Fig. 5. The result of Genotype CM (line a) and AS (line b) kit line a): positive bands show *M. ulcerans-M. marinum,* line b): positive bands show *M. ulcerans*

hsp65gene Sequencing: A total of 441bp of the hsp65gene was sequenced (CEQ8000 Sequence Analysis System, Beckman-Coulter, USA). The obtained sequence was compared to those stored in GenBank using the Basic Local Alignment Search Tool (BLAST; NCBI, Bethesda, MD) and was shown to be 99% homologous to the *Mycobacterium marinum* ATCC927 *hsp65* gene sequence deposited under accession number AF476470.

Histopathology: The most prominent histological changes were seen in the visceral organs and gills. The hematoxylin-eosin (H&E) stained sections showed multifocal well-formed epithelioid cell granulomas in spleen, liver, kidney, heart, and fibrose tissue of gill cartilage and gill filaments. Granulomas were composed of concentric layers of epithelioid cells forming a discrete spherical lesion (Fig. 6a,b,c,d). Significant variations in size and structural organization of granulomas was observed from highly organized lesions with thick epithelioid layers to early stage of granuloma formation that poorly organized inflammation with minimal epithelioid cell formation. Caseous necrosis of the core region nodules were usually observed in the visceral organs. Giant cells were not observed in these spontaneously originated granulomas. Granulomatous lesions with acid fast rods (AFRs) were found in all affected fish and were localized in the spleen, liver kidney, heart and fibrose tissue of gill arch cartilage and gill filaments (Fig. 7a,b,c,d). The affected fish gill arch cartilage had granulomatous inflammation in the connective tissue. In addition to submiliary and miliary granulomas, the formation of coalescing granulomas

caused by fusion of granulomas was observed, and their expansion destroyed entire organs.

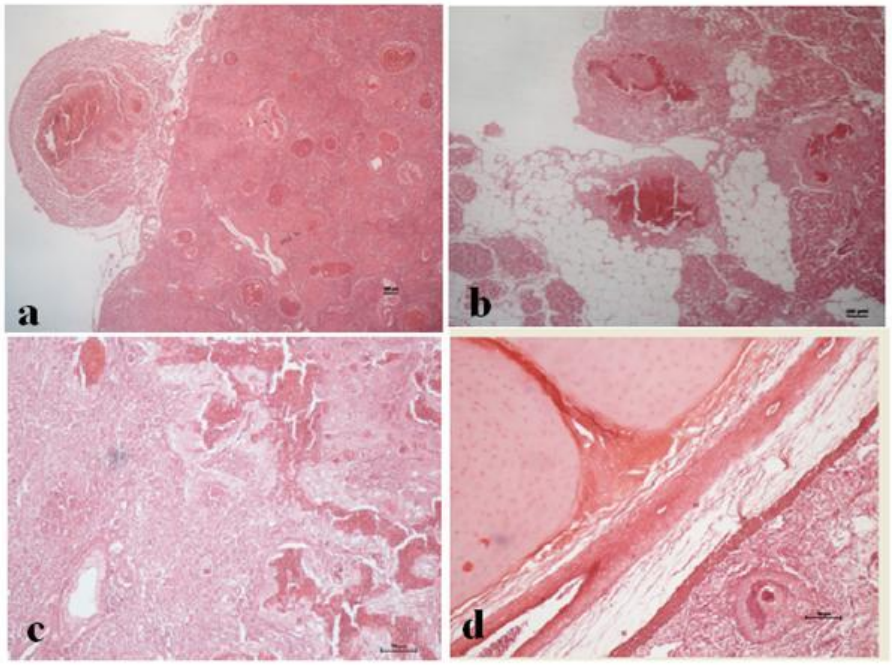

Fig. 6. Multifocal epithelioid cell granulomas
(a) in spleen,
(b) in liver,
(c) in kidney,
(d) on the fibrose tissue of gill arch cartilage (H&E)

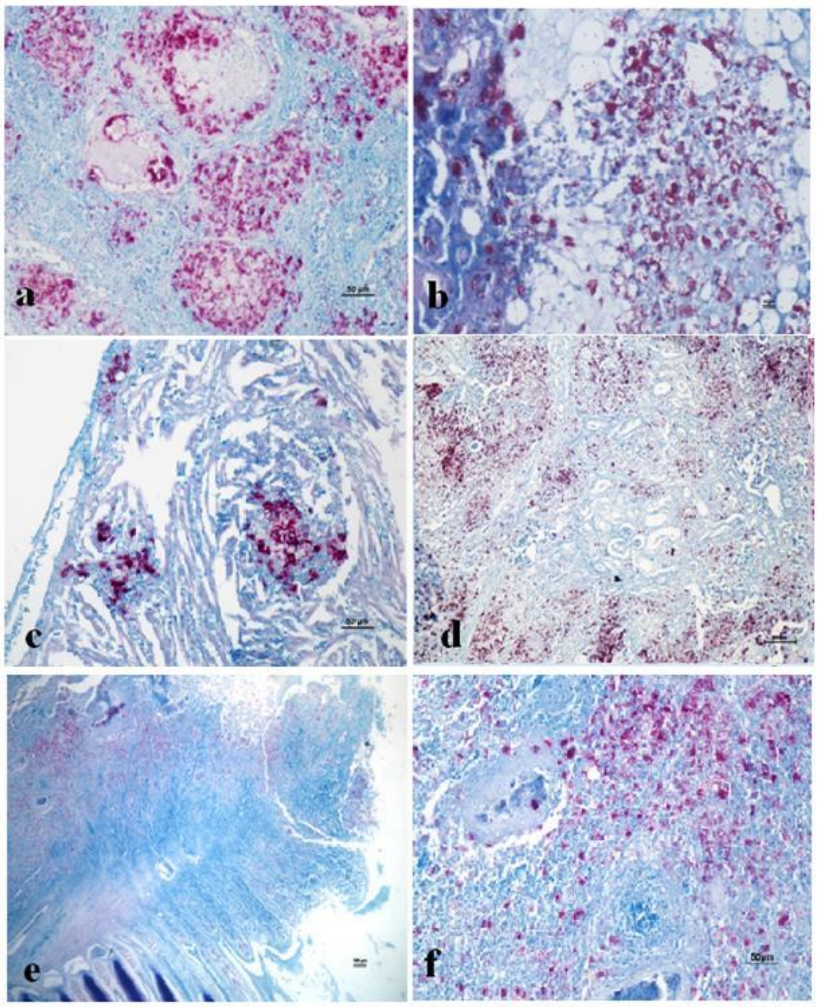

Fig. 7. Visceral organs of meagre with granulomas containing acid fast rods (Ziehl-Neelsen)
(a) in spleen,
(b) in liver,
(c) in heart,
(d) in kidney,
(e) in fibrose tissue of gill cartilage,
(f) in gill filament

Discussion

Mycobacteriosis was first identified in carp in 1897, and has since become the most common chronic disease affecting both cultured and wild freshwater fish, sea fish, aquarium fish (Amlacher, 1970; Arakawa & Frayer, 1984; Hedrick et al., 1987; Colorni, 1992; Colorni et al., 1996; Diamant et al., 2000; dos Santos et al., 2002; Toranzo et al., 2005) and occasionally humans (Ucko and Colorni, 2005). In cultured fish mycobacteriosis was reported in Pacific and Atlantic salmon, turbot, tilapia, and European sea bass. Mycobacteriosis caused by *M. marinum* is still a significant threat especially for sea bass cultured in the Mediterranean coasts of Greece, Israel, Italy and Turkey and the Red Sea Coast of Israel (Colorni, 1992; Colorni et al., 1993; Colorni et al., 1996; Diamant et al., 2000; Ucko et al., 2002).

In this study, a presumptive diagnosis of mycobacteriosis in marine cultured affected meagre was based on the observation of macroscopic white granulomas on the visceral organs and ZN positive short rods within the fresh squash mounts of the granulomas. Diagnosis was confirmed by the culture of microorganisms from the kidney, spleen, and liver of affected fish by using selective Lowenstein-Jensen medium, which produced slow growing yellow to orange colored colonies of the causative organisms as described in previous reports (Diamant et al., 2000; dos Santos et al., 2002; Rhodes et al., 2004; Jacobs et al., 2009; Novotny et al., 2010).

The isolates were identified to the species level using conventional methods (phenotypic characteristics), Genotype Mycobacterium AS and CM assay, or *hsp65* gene sequence data (Ucko et al., 2002, Richter et al., 2006; Gitti et al., 2005; Pourahmad et al., 2009).

The isolates were identified as *M. marinum* using the conventional phenotypic characterization methods being strongly acid fast, ZN positive especially photochromogenic pigmented, and having smooth-hemispheric colonies of the bacilli (Koneman et al., 1992; Plumb and Hanson, 2011).

The GenoType CM assay, targeting the 23S rRNA gene region, provides simultaneous identification of 14 different mycobacterial species (Richter et al., 2006). In the present study, all isolates identified with the banding pattern of the Genotype CM assay, were found to be either *M. marinum* or *M. ulcerans*. According to the subsequent use of the Genotype AS, the isolates were further identified as *M. ulcerans*. The result of the Genotype CM assay failed to differentiate *M. ulcerans* from *M. marinum*. This and the minimal pathogenesis it presented may explain why *M. ulcerans* was not reported as a fish pathogen causing chronic experimental infection in Japanese medaka (*Oryzias latipes*) (Mosi et al., 2012). Some phenotypic characteristics of our isolates such as photochromogenic yellow pigment production, and a shorter incubation period, did not bear similarities to *M. ulcerans* which produces light buff colored or non-pigmented colonies in 6-12 weeks (Koneman et al., 1992). These isolates were further differentiated by a moleculer method known as *hsp65* gene sequence analysis. According to this method, isolates were later identified as *M. marinum.* This result confirmed the phenotypic characteristics of the isolates in the present study. The sequence obtained in this study is defined as GenBank accession number KM279677.

The gross pathology observed in our findings bear similarities to mycobacterial infections in other fish species such as in cultured sea bass (Hedrick et al., 1987), cultured striped bass (Hedrick et al., 1987), rabbit fish (Diamant et al., 2000), European sea bass (Colorni, 1992; Colorni et al., 1996; Korun et al., 2005), sea bream (Colorni et al., 1996), farmed turbot (dos Santos et al., 2002), in striped bass from Chesapeake Bay (Rhodes et al., 2004) and in ornamental fish (Novotny et al., 2010). The most striking similarity is the various sized multifocal granulomas (2-7 mm) on the spleen, liver and kidney. Only one fish showed hemorrhagic ulcerative skin lesions at the base of the caudal fin as described by Gauthier and Rhodes (2009). The histopathology also bore similarities to that observed in striped bass (Hedrick et al., 1987), experimentally infected sea bass (Colorni et al. 1998), wild rabbit fish (Diamant et al., 2000), turbot (dos Santos et al., 2002), cultured sea bass (Korun et al., 2005), and in ornamental fish (Novotny et al., 2010). However, granulomas were not observed in the gut and eye tissue (Diamant et al., 2000; Novotny et al., 2010).

In general the granulomas present in the infected fish showed the same structure as those described in other fish with mycobacterial infection, and in the histological sections of the visceral organs, the varying size granulomas with or without a necrotic core, surrounded by epithelioid cells with a large amount of acid fast rods demonstrated by ZN stain (Diamant et al., 2000; dos Santos et al., 2002; Korun et al., 2005; Novotny et al., 2010). Multinucleated Langhans type giant cells previously reported by Timur (1975) and Timur et al. (1977) in the early development stage of the experimentally induced granulomas in plaice by piscine mycobacteria, were not observed in the mature granulomas in the present study or in other studies (dos Santos et al., 2002; Novotny et al., 2010).

The present clinical signs, gross pathology and result of the histopathology, bacteriology, Genotype Assay and sequencing of *hsp65* gene results indicated that *M. marinum* caused chronic granulomatous infection in farmed meagre. The regular monitoring of bacterial infections in marine farmed fish populations reared in floating net cages on the coast of the Aegean sea in Turkey revealed that the *M. marinum* infection affected two year old meagre causing significant mortalities. Interestingly, *M. marinum* did not infect the gilthead sea bream (*Sparus aurata*) populations in the same cage farm. This may suggest that gilthead sea bream are more resistant than meagre to *M. marinum.*

Acknowledgements

The authors would like to thank Dr. O. Kaya Koksalan who performed the *hsp65* gene sequence analysis.

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