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Araştırma Makalesi / Research Article

Mycobacterium marinum Infection in European Sea Bass (*Dicentrarchus labrax*) in Brackish Water Conditions

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Abstract

Mycobacteriosis is a well-known zoonotic disease that affects both fish and human. The pathogen, *Mycobacterium marinum*, cause systemic and lethal disease in fish and can be observed in farmed and aquarium species. In this study, *M.marinum* infection was detected in European sea bass (*Dicentrarchus labrax*) with common clinical and pathological findings in brackish water conditions (3.92 ‰). Granulomas in the enlarged spleen and liver were observed. The pathogen was isolated on Löwenstein-Jensen (L-J) media and the molecular identification verified the causative agent as *M.marinum*. Several granulomas were detected in histopathological examination. European sea bass has one of the highest production numbers in Türkiye, and this study aims to present mycobacteriosis in detail with clinical and pathological effects on European sea bass from brackish water conditions.

Keywords: mycobacteriosis, aquaculture, European sea bass, granulomas

Acı Su Koşullarında Avrupa Deniz Levreklerinde (*Dicentrarchus labrax*) *Mycobacterium marinum* Enfeksiyonu

Öz

Mikobakteriyozis, hem balıkları hem de insanları etkileyen, iyi bilinen zoonotik bir hastalıktır. Patojen *Mycobacterium marinum*, balıklarda sistemik ve ölümcül hastalıklara neden olur, çiftlik ve akvaryum türlerinde gözlenebilir. Bu çalışmada, yaygın klinik ve patolojik bulgularla Avrupa deniz levreğinde (*Dicentrarchus labrax*) *M.marinum* enfeksiyonu acı su koşullarında (3.92 ‰) saptanmıştır. Deride ülserler, büyümüş dalak ve karaciğerde granülomlar gözlenmiştir. Patojen, Löwenstein-Jensen (L-J) besiyerinde izole edilmiş ve moleküler tanımlama ile de *M.marinum* olduğu doğrulanmıştır. Histopatolojik incelemede çok sayıda granülom saptanmıştır. Avrupa deniz levreği, Türkiye'de en çok yetiştirilen balık türlerinden biridir ve bu çalışma, Avrupa deniz levreğinde klinik ve patolojik bulgularla acı su koşullarından mikobakteriyozu ayrıntılı olarak sunmaktadır.

Anahtar Kelimeler: Mikobakteriyozis, su ürünleri yetiştiriciliği, Avrupa deniz levreği, granülom

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1. Introduction

"Piscine tuberculosis" or "fish mycobacteriosis" is a worldwide chronic progressive disease that affects several fish species and associated with multiple species of the genus *Mycobacterium* (Lansdell et al., 1993; Jacobs et al., 2009; Gauthier and Rhodes, 2009; Keller et al., 2018). Mycobacteria are acid fast, free-living, robust, non motile bacteria that are present in widely diverse environments such as in tap water and soil which affect animals and human (Das et al., 2018). The most common atypical species belonging to the genus is *Mycobacterium marinum* (*M. marinum*), which causes opportunistic infection in the human (Rallis and Koumantaki-Mathioudaki, 2007; Hashish et al., 2018). Infected fish develop skin ulcers, enlarged visceral organs, mostly spleen and kidney with multiple granulomas (Colorni, 1992; Ravid-Peretz et al., 2019).

The first description of *M. marinum* was released by Aronson (1926) from infected salt water fish (*Abudefduf mauritii*, *Micropogon undulatus*, *Centropristes striatus*) exhibiting similar symptoms with human tuberculosis (Das et al., 2018). Subsequently, the pathogen was reported from several fish species such as Atlantic salmon (*Salmo salar*) (Brocklebank et al., 2003), turbot (*Scophthalmus maximus*) (Dos Santos et al., 2002), striped bass (*Morone saxatilis*) (Hedrick et al., 1987), rabbitfish (*Siganus rivulatus*) (Diamant et al., 2000), gilthead sea bream (*Sparus auratus*) (Avsever et al., 2016), meagre (*Argyrosomus regius*) (Avsever et al., 2014, Timur et al., 2015) and sea bass (*Dicentrarchus labrax*) (Colorni, 1992, Korun et al., 2005, Avsever et al., 2016). Likewise, the disease agent is widespread in ornamental fish species that Slany et al. (2014) reported a percentage of 41.7 % abundance in aquarium fishes (*Ancistrus* sp., *Corydoras caudimaculatus*, *Helostoma temminckii*, *Paracheirodon axelrodi*, *Paracheirodon innesi*, *Poecilia reticulate*, *Poecilia sphenops*, *Pterophyllum scalare*, *Trichogaster trichopterus* and *Xiphoporus maculates*) when environmental samples showed only 19.3% presence of bacteria. The reason of the high incidence of the pathogen was attributed to aquarium conditions for bacteria grow, high fish density, and frequent fish transfer between aquariums which provide suitable conditions for the spread of mycobacteriosis between fish species (Slany et al., 2014, Hashish et al., 2018).

M. marinum is reported as the most common "non-tuberculous mycobacteria" (NTM), which is piscine-related and results in granulomatous inflammation, especially on hands and fingers in the human (Jacobs et al., 2009). The infection in humans is usually known as "fish tank or swimming pool granuloma" based on the presence in still water sources like swimming pools or fish ponds that not treated with chlorine (Peña Merino et al., 2020) as well as transmission via direct contact with fish such as preparations or processing of seafood (Clark et al., 1990; Lawler 1994; Hashish et al., 2018; Kent et al., 2006). The origins of *M.marinum* infection cases in humans were reported in France as 84 % (53 patients) of exposure to indoor or outdoor aquariums, 5 patients for injury / contact with

oyster or fish spine, 1 patient from swimming pool and 4 patients infected with it from unknown sources (Aubry et al., 2002).

European sea bass is one of the main cultured fish specie in Turkey and the most produced fish in the Mediterranean area (Ferreira et al., 2010, Ulusoy and Mol, 2022). The European sea bass production has received 152.469 tonnes in 2022 (TUIK, 2023) and the prevelance of the bacterial disease agents are essential problem for producers.

In this study, *M.marinum* infection in Europeas sea bass (*Dicentrarchus labrax*) cultured in brackish water was observed and investigated in detail with obtaining of the pathogen, molecular identification, and determining of clinical and histopathological effects on diseased fish.

2. Materials and Methods

2.1. Fish samples

The fish were observed in brackish water conditions (3.92 ‰) of a recirculated aquaculture system (pH: 7.7, oxygen: 10.11 mg/L, temperature: 23 ºC). A total of infected 30 fish (approximately 250 g), recently dead European sea bass (*Dicentrarchus labrax*) were subjected to clinical, parasitological, microbiological, and histopathological investigations. Bacterial isolations from the kidney, spleen and liver of infected fish were streaked onto Löwenstein-Jensen (L-J) media and Triyptic Soy Agar (TSA) according to Austin and Austin (2007). The L-J media were incubated at 30 ºC for 2 weeks while TSA plates were incubated for colony forming at 21 ºC. The bacteria on L-J media was stained with Ziehl-Neelsen protocol (Arda, 2006).

22 Moleculer identification of pathogen

16S rRNA gene amplification of isolated bacteria was accomplished for molecular identification. EurXGeneMATRIX Bacterial & Yeast DNA Isolation Kit (Poland) was used for DNA isolation according to the manufacturer's instructions. The density and quality of isolated DNA was determined using spectroscopic measurements with Thermo Scientific Nanodrop 2000 (USA). The 27F (5' AGAGTTTGATCMTGGCTCAG 3') and 1492R (5' TACGGYTACCTTGTTACGACTT3') universal primers were applicated for PCR amplifications and the reactions were carried out as follows: initial denaturation at 95 ºC for 5 minutes, 40 cycles at 95 ºC followed by 45 seconds denaturation, 57 ºC-45 seconds for annealing, 72 ºC-60 seconds for extension and 72 ºC-5 minutes as the final extension. The amplification results were verified with 1.5 % agarose gel prepared with 1X TAE buffer at 100 volt for 90 minutes electrophrosis. The band screening was observed by using

ethidium bromide dye under UV light. One-step PCR was performed to amplify the region of 1470 and 700 bases, approximately. The PCR reaction was performed with Solis Biodyne (Estonya) FIREPol® DNA Polymerase Taq polymerase enzyme. It was observed that the PCR process was successful in obtaining a single band on agarose gel. During the purification step of the PCR product, the obtained single band samples were purified by using MAGBIO "HighPrepTM PCR Clean-up System" (AC-60005) purification kit according to the manufacturer's instructions. For Sanger sequencing, the ABI3730XL Sanger sequencing device (Applied Biosystems, Foster City, CA) and BigDye Terminator v3.1 Cycle (Applied Biosystems, Foster City, CA) were used in the Macrogen Netherlands laboratory. In BioEdit software, CAP contig assembly algorithm was used to form a consensus sequence based on the data obtained with primers 27F-1492R. The sequenced data were compared with using the BLASTN 2.6.1 from GenBank database. The Mega 7 software were used for the construction of the phylogenetic tree with neighbor-joining method (MEGA11: Molecular Evolutionary Genetics Analysis version 11 (Tamura, Stecher, and Kumar 2021).).

2.3. Histopathology

The histology of sampled tissues was performed according to Culling et al. (1985). After necropsy, the liver and spleen sections were preserved in 10% buffered formalin from diseased fish. The tissue samples were processed, embedded in paraffin blocks, sectioned at 5 µm thickness and stained with haematoxylin and eosin (H&E). The sections were examined under light microscope (Olympus CX22).

3. Findings and Discussion

M.marinum is an opportunistic pathogen that commonly isolated from saltwater, freshwater and brackishwater fish species (Ucko et al., 2002; Ziklo et al., 2018) associated with the symptoms of skin ulceration, granuloma in spleen, kidney and liver in fish (Hashish et al., 2018). Recently, similar clinical and pathological symptoms were confused with "pseudopasteurellosis" (*Aeromonas veronii* infection) in European sea bass (Tanrıkul and Dinçtürk, 2021), however the present study confirms the clinical and pathological effects of the disease on European sea bass with isolation and identification of the disease agent, *M.marinum* even detected with similar clinical signs. The infection was observed as chronic progression and the cumulative mortality was calculated as 23 %.

Bacteriological examinations and molecular identification of the disease agent were reported with histopathological effects on diseased *D.labrax* samples were reported in this study. The primary symptoms on the sampled fish clinically had skin lesions and necrotic areas on the body surface in

the present study (Figure 1 A, B, C, D). In addition, no parasitic agent was found during the examination. Keller et al. (2018) described clinical signs consisting of apathy, anorexia, and skin ulcerations. Avsever et al. (2016) observed skin lesions, cachexia and paleness in diseased fish. The infected sea bass did not show any gross external signs in the study of Colorni (1992), however fistulous perforation between the pectoral fins and unilateral exophthalmia in one specimen were observed. Korun et al. (2005) reported pale gills, exophthalmia and fin hemorrhage in affected sea bass as clinical signs of the disease.

In the necropsy, tubercules were observed in the internal organs, especially diffuse granulmoas were seen in the spleen and liver (Figure 1 E, F, G, H). The primary pathological sign of *M.marinum* in fish is reported as classical granulomatous inflammation (Roberts, 2001; Jacobs et al., 2009). It was reported that acute forms of *M.marinum* infection were characterized by high mortality, however, granuloma in different organs formed between 4-8 weeks (Van Der Sar et al., 2004; Hashish et al., 2018). Unlikely, Jacobs et al. (2009) assumed that granulomas were found during early stages, but than spread to internal organs in more advanced cases. Tubercule formation and enlargement of the spleen (Avsever et al., 2016), nodular lesions in liver, kidney, and spleen (Hedrick et al., 1987) were reported as the most common pathological findings in Mycobacteriosis cases.

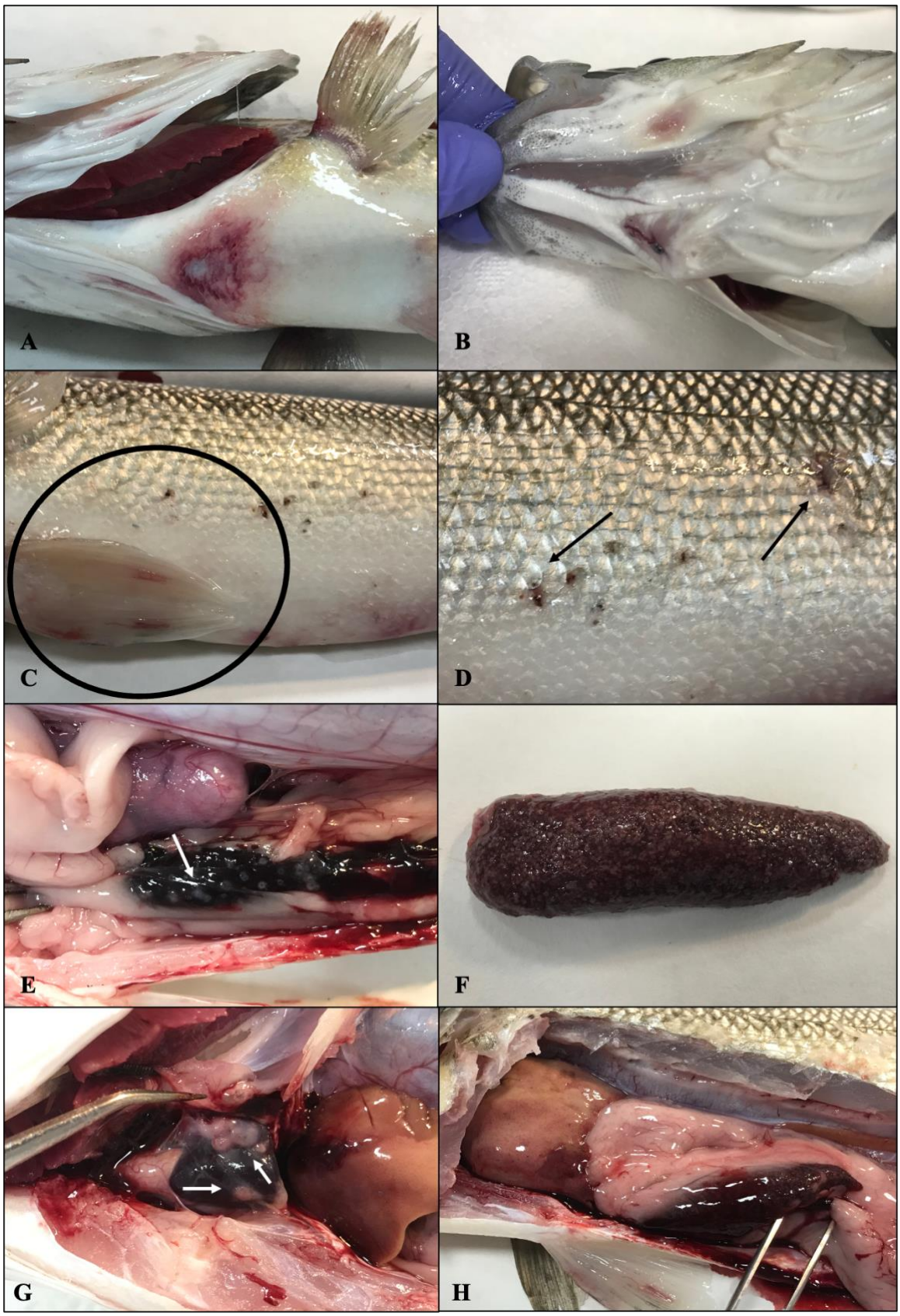

Figure 1. A-B: Necrotic areas on the ventral region, C: hemorhages in the ventral fins, D: different grades of skin lesions, E-F: tubercules in the spleen, G: granulomas in heart, H: enlarged spleen and liver, diffuse hemorrhage in liver

The diagnosis of mycobacteriosis in fish is mainly based on gross examination of internal organs that may show white granulomatous nodules, however, culturing the bacteria on Middlebrook 7H10 or Löwenstein-Jensen media (L-J media) is an important method for diagnosis and for further biochemical and molecular identification (Jacobs et al., 2009). There was no colonies formed on TSA in the microbiological studies, on the contrary, yellow colonies were detected after 20 days of inoculation on L-J media from the inoculations of the kidney, spleen and liver of diseased fish (Figure 2A).

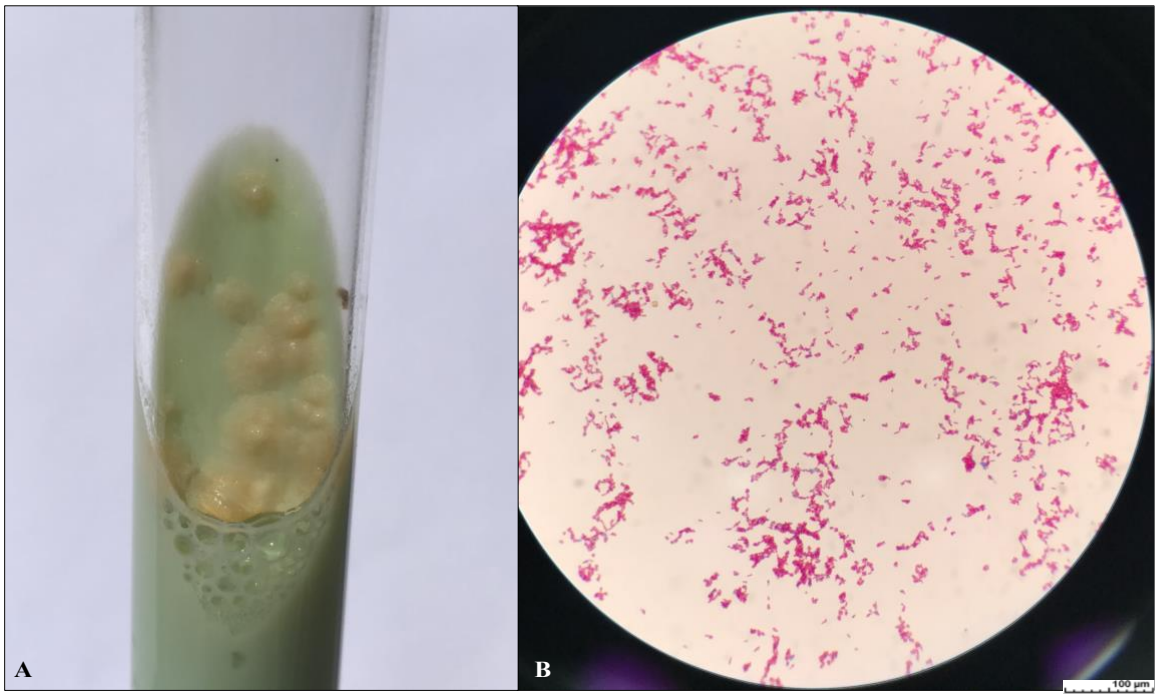

Figure 2. A: Yellow colony formed on Löwenstein-Jensen media, B: Ziehl-Niellsen stained *M. marinum* under light microscopy (immersion x1000)

The isolated strain showed 99% similarity with *M. marinum* based on 16S rRNA sequence results in the BLASTN 2.6.1 database and the sequence results were registered with the accession number OM319833 in NCBI GenBank. The phylogenetic tree belonging to the isolated *M.marinum* strain and related homologous sequences that obtained from GenBank was presented in Figure 3. Das et al (2018) reported that *M.ulcerans* and *M.marinum* are the closest neighbours in phylogenetic analyses depending 16s rRNA sequence results, and *M.marinum* is also a member of *M.ulcerans* clade which means *M.ulcerans* and *M.marinum* are together constituting the *M.ulcerans-M.marinum* complex (Aronson, 1926; Qi et al., 2009; Pidot et al., 2010; Das et al., 2018; Hashish et al., 2018). Ucko et al. (2002) determined that *M.marinum* isolates from geographically different sources showed divergence based on restriction enzyme maps depending on the nucleotide sequences. Comparative sequence analyses were recommended to determine the epidemiology and evolution of the strains to be beneficial both for fish and human cases (Kurokawa et al., 2013; Hashish et al., 2018).

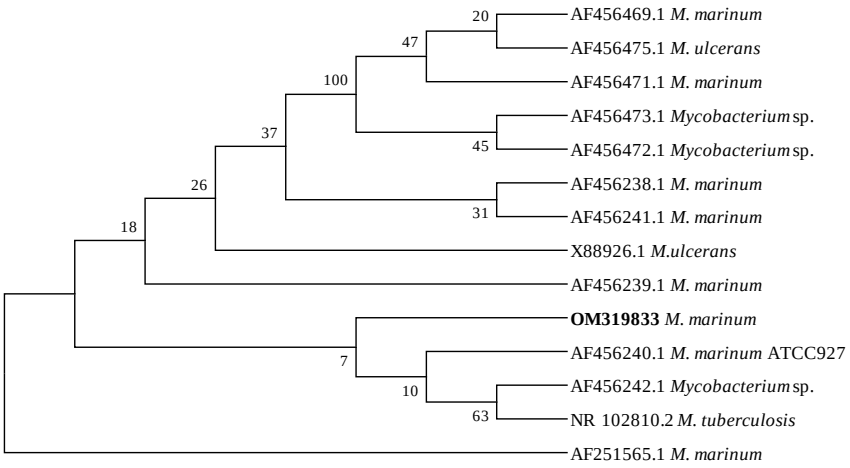

Figure 3. Phylogenetic tree of *Mycobacterium marinum* OM319833 (in bold) and related matches in Gen-Bank (NCBI)).

In the histopathologic examination of the infected spleen and liver, several granulomas were present caused by mycobactertosis. Numerous epithelioid cell granulomas were detected on spleen tissue (Figure 4A). The mycobacteria clumps appeared inside the nodules as the cause of bacterial inflammation (Figure 4B). Similarly, nodule appearance was clearly discerned on the surface of the liver surrounded by inflammatory cells (Figure 4C). There are similarities between the present results and previous observations of mycobacterium infection in fish. Avsever et al. (2014) observed granulomas surrounded by inflammatory cells in the kidney, liver, and spleen tissues of infected meagre (*Argyrosomus regius*). Ostland et al. (2008) reported that spleen and head kidney were more severely affected than liver and chronic inflammation was stated as the predominant histopathological change. These results are likely to be related to Diamant et al. (2000). The various stages of scattered granulomata appeared multifocal necrotic debris in the splenic pathology of infected rabbitfish (*Siganus rivulatus*). Colorni et al. (1998) indicated histopathological examination of experimentally infected sea bass with *M.marinum* and the results displayed similar disease patterns in liver and spleen tissues.

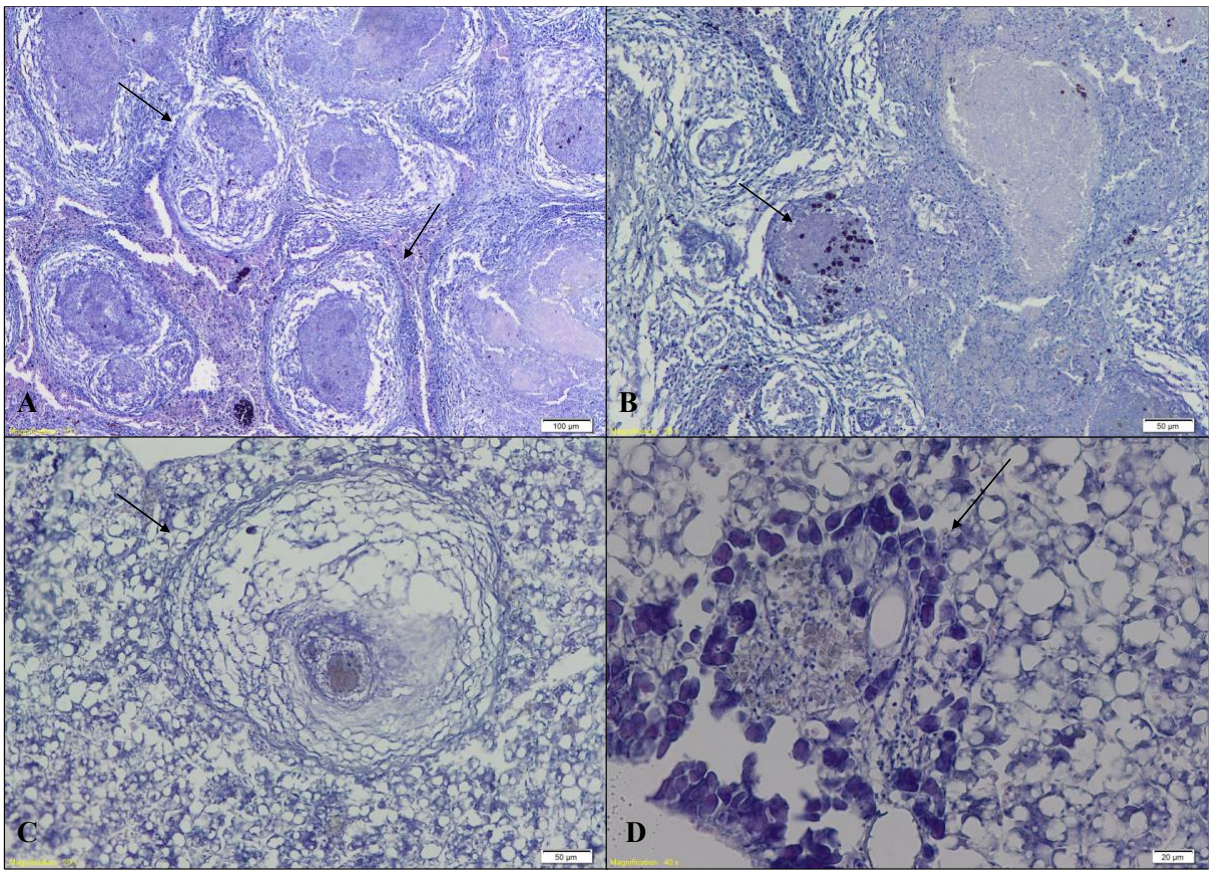

Figure 4. A: Multiple granulomas in spleen (x10), B: Bacteria clumps in splenic lesion (x20), C: Nodule development on the surface of liver (x20), D: Inflammatory cells caused by mycobacteria in liver tissue (x40) (H&E)

4. Conclusions and Recommendations

This study reveals a detailed investigation of *M.marinum* infection in European sea bass from brackish water conditions. Due to its zoonotic potential, it is important to isolate and identify the pathogen and present the clinic and pathogenic results of one of the most cultured fish species, European sea bass.

Authors' Contributions

All authors contributed equally to the study.

Statement of Conflicts of Interest

There is no conflict of interest between the authors.

Statement of Research and Publication Ethics

The author declares that this study complies with Research and Publication Ethics.

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