Diagnosis of *Micrococcus luteus* Infection in Cultured Sharpsnout Sea Bream (*Diplodus puntazzo* Cetti, 1777)

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Abstract: The aim of this study was to determinate the causative agent of mortalities in sharpsnout sea bream (*Diplodus puntazzo* Cetti, 1777) cultured in the Aegean Sea. Ten individuals (200-350 g) in were investigated total between May 2009 – 2010 using bacteriological methods. Moribund fish samples exhibited pale gills and liver, skin depigmentation, hemorrhages on the liver and muscles. Bacteriological samples of liver, kidney and spleen were streaked onto Marine Agar (MA) and Tryptic Soy Agar (TSA) plates containing 1,5% NaCl and incubated at 22°C for 48h. Gram-positive non-motile cocci were isolated from visceral organs of diseased fish samples. According to their morphological and biochemical characteristics, these isolates were identified as *Micrococcus luteus*. Kirby-Bauer disk diffusion method was also performed using multidisc. Results were interpreted based on the available data and all isolates were determined to be sensitive against oxytetracycline, erythromycin and chloramphenicol. Histopathologically, vacuolar degeneration and necrosis in the parenchyma cells of liver, tubular necrosis in the kidney and hemosiderin deposite in the spleen were determined. In addition to melting lamella, epithelial cells hyperplasia in the gills was observed. In conclusion, in this study an epizootic with a low mortality rate caused by *M. luteus* was described and this study is the first report of the pathogen in the cultured sharpsnout sea bream.

Kültüre Edilen Sivriburun Karagöz Balıklarında (*Diplodus puntazzo* Cetti, 1777) *Micrococcus luteus* Enfeksiyonunun Teşhisi

Anahtar Kelimeler
Sivriburun Karagöz
Kafes Yetiştiriciliği,
Micrococcus luteus

Öz: Bu çalışmanın amacı Ege Denizi'nde kültüre edilen sivriburun karagöz balıklarında (*Diplodus puntazzo* Cetti, 1777) meydana gelen ölümlerin nedeninin belirlenmesidir. Mayıs 2009 ve Mayıs 2010 tarihleri arasında bakteriyolojik yöntemler kullanılarak toplam 10 hasta birey (200-350g) incelendi. Ölmek üzere olan balık örneklerinde solgun solungaç ve karaciğer, deri renginde açılma, karaciğerde ve kaslarda kanamalar görüldü. Hasta balıkların karaciğer, böbrek ve dalağından Marine Agar (MA) ve % 1,5 NaCl içeren Tryptic Soy Agar'a (TSA) yapılan bakteriyolojik ekimler 22 ° C'de 48 saat süreyle inkübe edildi. Gram pozitif hareketli olmayan koklar viseral organlardan izole edildi. Morfolojik ve biyokimyasal özelliklerine göre, bu izolatlar *Miccrococcus luteus* olarak identifiye edildi. Kirby-Bauer disk difüzyon yöntemi çoklu disk kullanılarak yapıldı. Sonuçlar mevcut verilerine dayanılarak yorumlandı ve tüm izolatların oksitetrasiklin, eritromisin ve kloramfenikole karşı duyarlı olduğu belirlendi. Histopatolojik olarak karaciğer parankim hücrelerinde vakuolar dejenerasyon ve nekroz, böbrekte tübüler nekroz ve dalakta hemosiderin depozitleri saptandı. Lamellalarda erimenin yanı sıra solungaçlarda epitel hücre hiperplazisi gözlendi. Sonuç olarak, bu çalışmada *M. luteus*'un neden olduğu düşük mortaliteli bir epizootik tanımlandı ve bu çalışma bu patojenin kültür sivriburun karagöz balıklarında tanımlandığı ilk rapordur.

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1. Introduction

Sharpsnout sea bream, *Diplodus puntazzo* (Cetti, 1777) is a marine fish species distributed in the eastern Atlantic Ocean, Mediterranean sea and Adriatic sea [1]. This fish species has been successfully introduced to aquaculture in the Mediterranean since 1990's due to its high market value [3-5]. In Turkey, first sharpsnout sea bream aquaculture has begun in 2000's in the Aegean Sea. Brood stock management, embryonic development stages and larviculture of this fish were studied and described by Kamacı et al.; Kop et al. and Suzer et al. [6-8]. Total sharpsnout sea bream production of Turkey was 59 tonnes in 2015 [9]. Sharpsnout sea bream also has high mortality rates in on-growing facilities, mainly due to diseases and breeding problems of larvae [4, 5, 10]. Up to day, various infectious and non-infectious diseases were described in this vulnerable cultured species. *Aeromonas hydrophila* [10, 11], *Photobacterium* subsp. *piscicida*, *Vibrio alginolyticus*, *V. splendidus*, *V. vulnificus, Staphylococcus epidermidis* [10] and *Edwardsiella tarda* [12] were isolated as a causative agent from cultured sharpsnout sea bream in Greece. In Turkey, a Gram-positive pathogen, *Staphylococcus capitis* subsp. *capitis*, has been reported by Çanak and Timur [13].

The members of the genus *Micrococcus* which is included under the family usually consist of non-spore, aerobic, immobile, quaternary and irregular clusters of Gram-positive cocci. Most of the members that react positively in the catalase test and produce carotenoid pigment [14-15]. They live in various environments, inhabit the soil and may occur in several other ecological niches such marine sediment, chicken meat, fresh water or food as well as in mammals [16-18]. Therefore, they also described emerging opportunistic pathogens [17, 18], some of them are also used as probiotics in aquaculture [19-21]. Micrococcosis was first detected by Conroy in 1966 from rainbow trout (*Oncorhyncus mykiss*) [22], then it was reported that the disease caused high mortalities in rainbow trout fry (0,5-5 g) in England [17]. *M. luteus* was demonstrated as a causative agent by Austin and Stobie [17] and moribund fish exhibited exophthalmia, pale gills, enhanced skin pigmentation, swollen abdomen, swollen kidney, pale and elongated spleen and accumulation of acidic fluid [17]. In Turkey, the disease was reported by Aydın et al. [23] in rainbow trout in Erzurum region in freshwater environment. Also it was reported as a pathogen of cultured gilthead sea bream (*Sparus aurata*) [24] and common dentex (*Dentex dentex*) [25] in the Aegean coasts. The aim of this study is to determinate the causative agent of low mortalities observed in sharpsnout sea bream cultured in the Aegean Sea.

2. Material and Methods

2.1. Fish Samples

In this study, ten moribund (250-350g in weight) individuals were investigated between May 2009 and May 2010. The behaviours of the moribund fish samples were monitored and the anamnesis information of the epizootic was obtained from the fish farm executives. The fish samples, standing still on the water surface that exhibited clinical symptoms were collected from the cages, and autopsy was performed according to Noga [26] and Bullock [27].

2.2. Bacteriological Examination

Totally 10 moribund sharpsnout sea bream individuals were investigated by using bacteriological methods. Samples from internal organs were streaked onto Marine Agar and Tryptic Soy Agar (containing 1,5% NaCl) and incubated at 22°C for 48h. After incubation, the isolated pure bacterial cultures were examined using standard laboratory protocols and identified through biochemical characterization according to Kocur et al. [16] and Buller [28]. Also a rapid diagnosis kit API Staph (Biomeriux, France) was used according to the directions of the producer was used for all isolates.

2.3. Antimicrobial Susceptibility Testing

All isolates were tested for antimicrobial susceptibility by using Kirby-Bauer disc diffusion method including multidisc (chloramphenicol, erythromycin, oxytetracycline, kanamycin, streptomycin, ampicillin, ciproflaxacine and flumequine). Isolates were streaked onto Mueller-Hinton agar (Oxoid), incubated at 22°C for 48h, susceptibility zone diameters were measured and analyzed according to Clinical and Laboratory Standards Institute (CLSI) [29-30].

2.3. Histological Examination

Histopathological tissue samples (approximately 1 mm^3) from the internal organs were taken and fixed in 10% buffered formalin, processed with routine methods and embedded in paraffin blocks. Sections (5 µm) were stained with hematoxylin-eosin (HE) [31-32] and examined under light microscope using the image analysis system NIS-Elements BR Microscope Imaging Software (Nikon Instruments).

3. Results

Moribund fish samples exhibited pale gills and liver, loss of scales, lysis of fins and skin depigmentation (Fig. 1a), hemorrhages on the pale liver and muscles, splenoatrophy and accumulation of a mucoid yellowish fluid in the intestines (Fig. 1b).

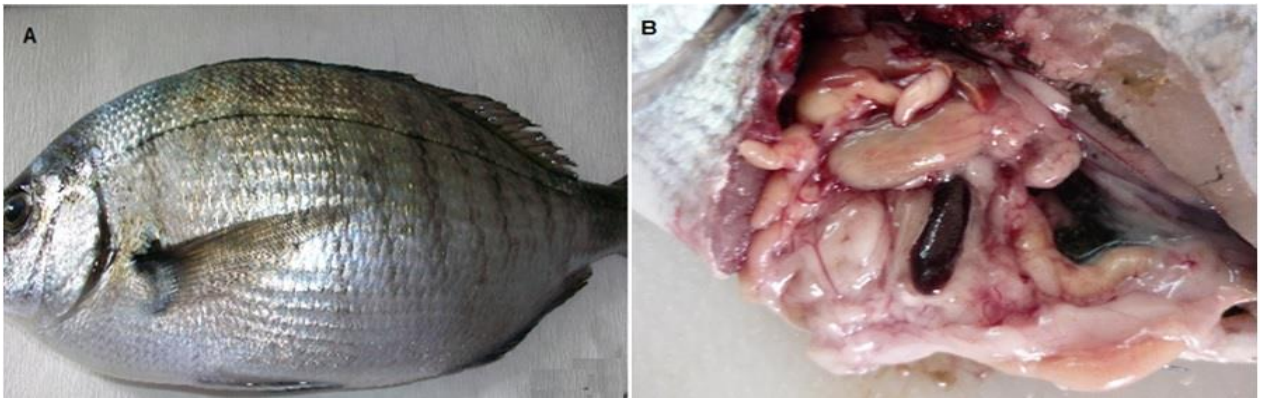

Figure 1. Moribund fish exhibited (A) skin depigmentation, scale loses, lysis of fins, (B) hemorrhages in the liver, mucoid yellowish fluid in the intestine.

Total of 10 yellow-pigmented Gram-positive isolates were recovered from kidney, spleen, liver and blood of moribund fish samples (Fig. 2a, b). All isolates exhibited the same biochemical and morphological characteristics with, only difference in a few biochemical tests such as arginin dihydrolase, Voges-Prouskauer, citrate and urease utilization. And all of them were identified as *M. luteus* (Table 1). API Staph results of these isolates were also showed in Figure 3. In the antimicrobial susceptibility test, all isolates were determined to be sensitive against oxytetracycline, erythromycin and chloramphenicol.

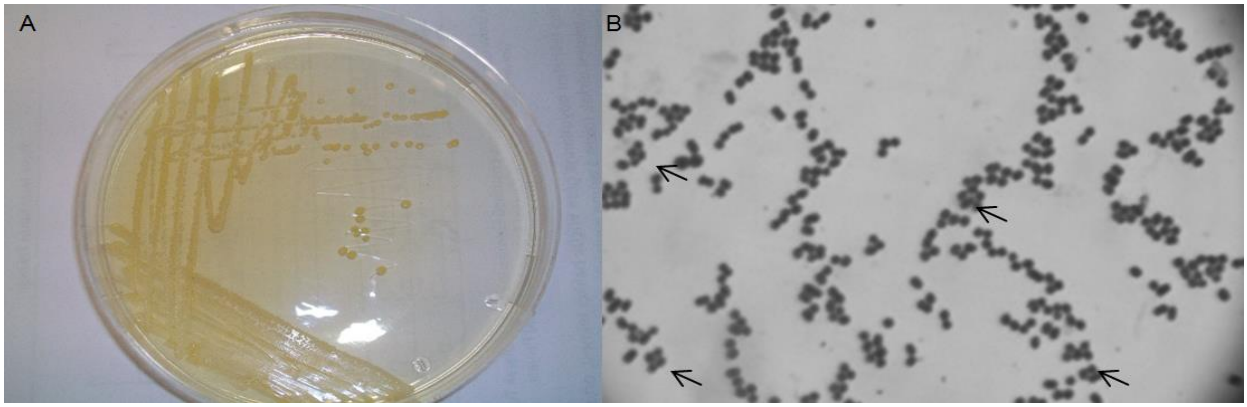

Figure 2. Isolated bacteria (A) yellow-pigmented colonies, (B) Gram-positive result of Gram staining and characteristic tetrad arrangement (arrowed).

Tablo 1. Phenotypic and morhpological caracteristics of *M. luteus* isolated from cultured sharpsnout sea bream

Characteristics	*M. luteus* (n:10)
Colony colour	Yellowish
Morphology	Cocci
Gram Staining	+
Motility	-
Cytocrom Oxidase	+
Catalase	+
Coagülase	-
O/F Test	+/-
0/129 (150µg)	R
Indole	-
Metil Red	-
Voges-Prouskauer Test	V
Arjinin	V
Lizin	-
Ornitrin	-
ONPG	-
Inositole	-
Arabinose	-
Sorbitole	-
Production of H_2S	-
Citrate	V
Urease	V

+:pozitive reaction, -: negative reaction, R: resistant, V: variable result

Figure 3. API Staph profile of *M. luteus* isolates

Histopathologically, vacuolar degeneration and necrosis in the parenchyma cells of liver (Fig. 4a) and hemorrhage in the liver (Fig. 4f), tubular necrosis in the kidney (Fig. 4b), hyperplasia of intestinal mucosa and hemorrhages in the intestines (Fig. 4c), multifocal hemosiderin deposits, degeneration of parenchyma cells and reduced hemopoietic tissue in the spleen were determined (Fig. 4d). In addition to melting lamella tissue, epithelial cells hyperplasia in the gills was also observed (Fig. 4e).

4. Discussion and Conclusion

In aquaculture, increased production, pressures on faster growth, high density, management and structure efficiency can create conditions favorable for the outbreak of bacterial infectious diseases. The causative agents of these diseases are predominantly opportunistic pathogens. It is also related to a weakened immune system and an altered microbiota [18]. Gram-negative bacteria are still dominant in the pathology of bacterial fish diseases, but Gram-positive bacteria have also been observed in recent years [13, 17, 18]. The study describes the successful identification of *M. luteus* strains in infected fish using bacteriological and histopathological methods.

A few outbreaks of the disease caused by *M. luteus* were diagnosed by Pekala in rainbow trout and brown trout in Poland between 2014 and 2016 [18]. Diseased fish exhibited mainly exophthalmia, skin melanization, pale gills and spleen, swollen abdomen and kidneys. Furthermore, the researchers supposed that the bacteria would appear more often in the future causing considerable commercial losses [18]. However, we have observed a lower mortality rate in the sharpsnout sea bream in the present study. In this study, moribund fish samples exhibited pale gills and liver, skin depigmentation, hemorrhages in the liver and muscles. Çanak and Timur [13] also reported similar clinical findings in the fish infected with *Staphylococcus capitis* subsp. *capitis* in Turkey.

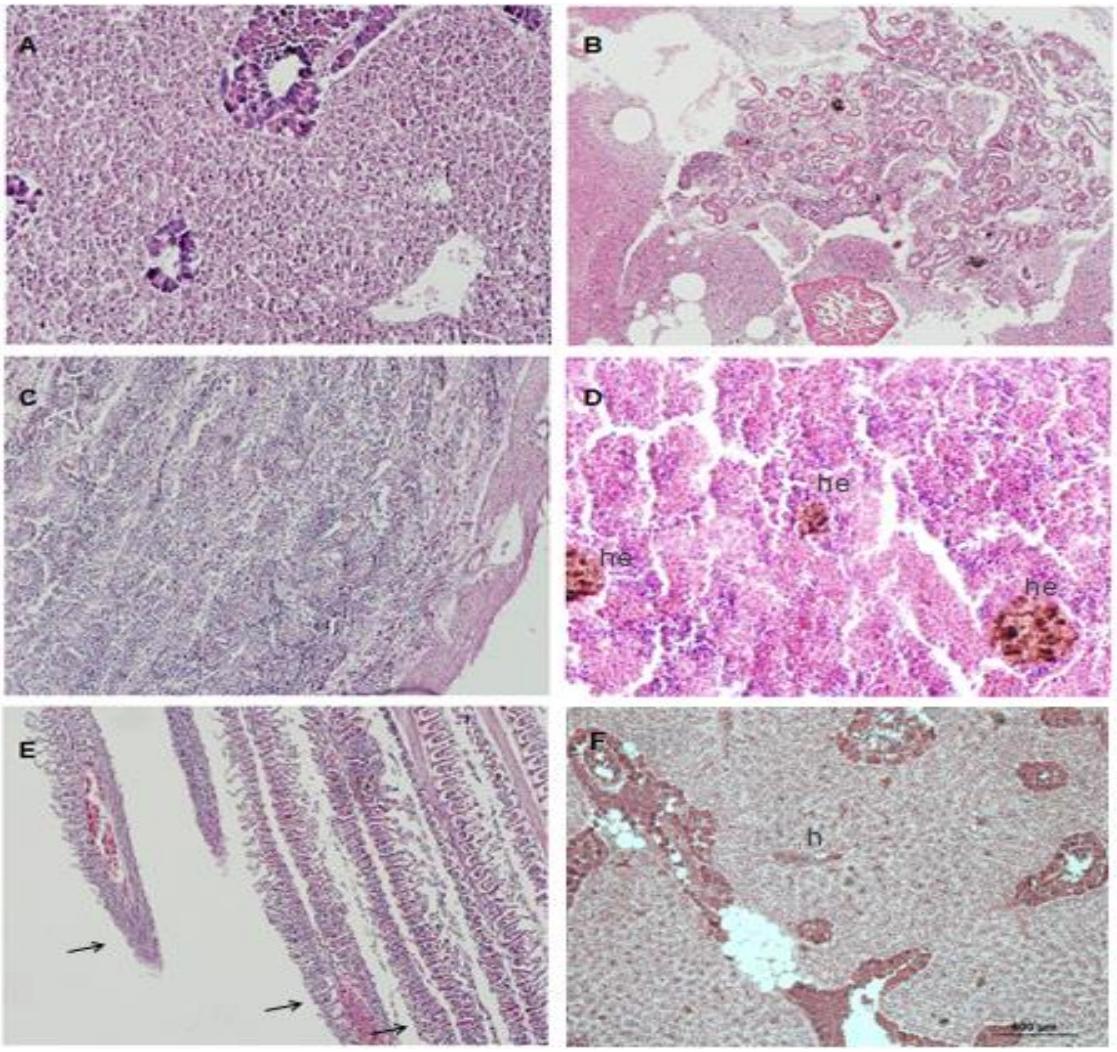

Figure 4. Tissue sections of moribund fish samples, stained with H&E. (A) vacuolar degeneration and necrosis in the parenchyma cells of liver, (B) tubular necrosis in the kidney, (C) hyperplasia of intestinal mucosa and hemorhages in the intestines, (D) multifocal hemosiderin deposits (he), degeneration of parenchyma cells and reduced hemopoietic tissue in the spleen, (E) hyperplasia of the gill lamellae (arrowed) and disruption of gill epithelium, (F) hemorrhagie (h) in the liver

M. luteus is a yellow-pigmented non-motile, oxidative bacterium with, Gram-positive cocci shaped cells, which display a characteristic tetrad arrangement. In this study, bacterial isolates recovered from moribund fish samples exhibited yellowish colonies, a tetrad arrangement, and the positive reaction of catalase. These bacteria did not produce ß-galactosidase, H_2S, indole, lysine or ornithine decarboxylase. The API Staph profile and other biochemical characteristics of all isolates were found to be similar to previously reported [17, 18, 22, 26]. This is the first report of M. luteus infection causing disease in cultured sharpsnout sea bream.

The sensitivity of the bacteria to chloramphenicol, streptomycin, potentiated sulphonamides and tetracycline was recorded by Austin and Stobie [17]. Some data concerning the antimicrobial resistance of *Kocuria, Micrococcus, Nesterenkonia, Kytococcus,* and *Dermacoccus* were presented by Szczerba without species specifications, and erythromycin susceptibilities were found [33]. In another study, inhibition zones were observed for enrofloxacin and sulphonamide with trimethoprim, besides flumequine and oxolinic acid resistance [18]. Previously, fish pathogenic *M. luteus* strains recovered gilthead seabream and common dentex were reported to be sensitive against oxytetracycline and erythromycin. In this study, all isolates were determined to be resistant against kanamycin, streptomycin, ampicillin, ciprofloxacin, and flumequine. Similarly to other studies, they were found to be sensitive to oxytetracycline, erythromycin, and chloramphenicol [18, 24, 25, 33].

In the present study, histopathological findings coincide with previously reported in the fish tissues infected with staphylococcal bacteria [13, 17, 18, 22, 26]. Our histopathological findings such as vacuolar degeneration and necrosis in the parenchyma cells of the liver and hemorrhage, tubular necrosis in the kidney, multifocal

hemosiderin deposits in the spleen, melanomacrophage deposit and necrosis of the kidney tubules are similar to the findings obtained by Çanak and Timur [13] in a staphylococcosis case of cultured sharpsnout sea bream *M. luteus* and *Vibrio* sp. .

In conclusion, Gram-positive, strictly aerobic coccoid bacteria, especially *M. luteus*, are a part of the normal bacterial flora of fish. Some of these species are used as probiotics in fish [19, 34], but some others may become pathogenic under stress conditions. For this reason, these bacteria are renamed as emerging opportunistic pathogens as others researchers have suggested.

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