COMU J Mar Sci Fish 2(1): 132-138

Journal Home-Page: http://jmsf.dergi.comu.edu.tr
Online Submission: http://dergipark.gov.tr/jmsf

COMU JOURNAL OF MARINE SCIENCE AND FISHERIES

Research Article

Bacterial Infection in Cultured Common Dentex (*Dentex dentex*, L. 1758)

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(Received: 28.05.2019; Accepted: 28.06.2019)
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Abstract: The aim of this study is the identification of microbial agents recovered from 8 moribund common dentex (*Dentex dentex*) samples (140-200 g), determination of their antimicrobial susceptibilities and description of histopathological changes. Main clinical external and internal symptoms of bacterial haemorrhagic septicaemia cases such as fin base and liver haemorrhages, splenomegaly, accumulation of fluid in the intestines and peritoneal cavity were observed. Bacteriological samples of liver, kidney and spleen were streaked onto Marine Agar and Tryptic Soy Agar plates containing %1,5 NaCl and incubated at 22°C for 48h. Gram-positive non-motile cocci and Gram-negative motile bacillus were isolated from visceral organs. According to morphological and biochemical characteristics, isolates were identified as *Micrococcus luteus* and *Vibrio* sp. The Kirby-Bauer disk diffusion method was also performed using multidisc and all isolates were determined to be sensitive against kanamycin, oxytetracycline and flumequine. In the infected fish, general cell degenerations and necrosis in various tissues, a remarkable increase in the goblet cells of the intestines, hepatic lipidosis in the liver, hyperplasia in the gills and the presence of melanomacrophage centres in various tissues were demonstrated. As a result, here, a mixed infection in moribund fish samples was described and this is the first report on the histopathological changes of *M. luteus* infection in a marine fish species. This bacterium was detected as a possible pathogen of common dentex with various external, internal and histopathological damages.

Keywords: Fish diseases, Histopathology, Common dentex, *Micrococcus luteus*

Kültür Sinarit (*Dentex dentex*, L. 1758) Balıklarında Bakteriyel Enfeksiyon

Özet: Bu çalışmada toplam 8 adet hasta sinarit (*Dentex dentex*) (140-200g) balığının hastalık etkenlerinin identifikasyonu ve antimikrobiyal duyarlılıkları yanı sıra balıklarda meydana gelen histopatolojik değişimlerin ortaya çıkartılması amaçlanmıştır. Hasta balık numunelerinde, yüzgeç diplerinde ve karaciğerde hemorajiler, dalakta büyüme, bağırsaklar ile periton boşluğunda sıvı birikimi gibi bakteriyel hemorajik septisemi olgularında görülen iç ve dış semptomlar gözlemlenmiştir. Bakteriyolojik ekimler enfekte balıkların karaciğer, böbrek ve dalak gibi iç organlarından %1,5NaCl içeren Triptik Soy Agar'a ve Marine Agar'a yapılmış ve petri kapları 22°C'de 48 saat inkübe edilmiştir. İç organlardan Gram-pozitif hareketsiz koklar ve Gram-negatif hareketli basiller izole edilmiştir. Morfolojik ve biyokimyasal özelliklerine göre izolatlar *Micrococcus luteus* ve *Vibrio* sp. olarak identifiye edilmiştir. Kirby-Bauer disk diffüzyon metodu çoklu disk yöntemi kullanılarak uygulanmış ve tüm izolatların kanamisin, oksitetrasiklin ve flumekuine karşı duyarlı olduğu tespit edilmiştir. Enfekte balıkların çeşitli dokularında genel olarak hücre dejenerasyonu ve nekrozu, bağırsaklarda goblet hücrelerinde gözle görülür bir artış, karaciğerde hepatik lipidosis, solungaçlarda hiperplazi ve çeşitli dokularda melanomakrofaj merkezlerinin varlığı gözlenmiştir. Sonuç olarak; bu çalışma ile hasta deniz balığı örneklerinde karma bir enfeksiyon tanımlanmıştır ve bu bir deniz balığı türünde *M. luteus* enfeksiyonuna bağlı histopatolojik değişimler üzerine ilk rapordur. Bu bakterinin, neden olduğu çeşitli dış, iç ve histopatolojik etkileri nedeniyle sinarit balığının olası bir patojeni olduğu tespit edilmiştir.

Anahtar Kelimeler: Balık hastalıkları, Histopatoloji, Sinarit, *Micrococcus luteus*

Introduction

Common dentex (*Dentex dentex*, L. 1758) is a marine fish species with a high commercial value (Company et al., 1999) naturally distributed in Indo-Pacific Ocean, Eastern and Western Atlantic Ocean and Mediterranean Regions (FAO, 2018). It was also described as a strong candidate among new fish species currently cultivated in Turkey (Company et al., 1999; Saka et al., 2007). Common dentex is cultured in

Mediterranean countries (Greece, Italy and Spain) and in Turkey as well since 2000's (Rudea & Matinez, 2001; Saka et al., 2007) and the total common dentex production of Turkey was 51 tonnes in 2017 (TUİK, 2018). During cultivation, various bacterial, viral and parasitic infections of common dentex were described (Rigos & Katharios, 2010).

Stress-related *Vibrio* epizootics of cultured common dentex caused by various *Vibrio* species which belongs to the family Vibrionaceae were reported previously in Greece (Rigos et al., 1998) and Spain (Company et al., 1999; Sitja-Bobadilla et al., 2007). In Turkey, Akaylı et al. (2015) recovered *Vibrio scophthalmi*, *V. alginolyticus*, *V. vulnificus*, *V. mediterranei* and *V. splendidus I* from the internal organs of moribund adult common dentex samples of 100-250 g in a long-term monitoring study. Also a *V. harveyi* infection was observed after the transportation of juveniles from hatchery to the marine cages and high mortality rates were reported (Turgay & Karataş, 2016).

Previously, members of the genus *Micrococcus* were reported from the intestinal microflora of cultured common dentex larvae (Akaylı et al., 2015), seabass and seabream (Grisez et al., 1997) and wild populations of pikeperch (*Stizostedion lucioperca*) (Diler & Diler, 1998). *Micrococcus luteus* which belongs to the family Micrococcaceae is also an important member of microbial flora in water (Gomez et al., 2007). They produce yellow-pigmented colonies with non-motile, oxidative, Gram-positive cocci shaped cells displaying a characteristic tetrad arrangement (Austin & Austin, 2016). After it's first detection in 1966 in diseased rainbow trout (*Oncorhyncus mykiss*) (Conroy, 1966). *M. luteus* was reported as a causative diseases agent in rainbow trout (Austin & Stobie, 1992; Aydın et al., 2005; Türk et al., 2013), common dentex (Akaylı et al., 2015), gilthead seabream (Çanak & Akaylı, 2018) and sharpsnout seabream (Akaylı & Yardımcı, 2018). Besides, non-pathogenic strains of *M. luteus* were used as a possible probiotic-candidate against various important fish pathogens. Abd El-Rhman et al., (2009) reported its antagonism against *Aeromonas hydrophila* under *in-vivo* conditions, while Akaylı et al. reported antagonism against *Listonella anguillarum* (Akaylı et al., 2016a) and *Staphylococcus aureus*, *S. epidermidis* and *S. cohnii* subsp. *cohnii* (Akaylı et al., 2016b) under *in-vitro* conditions.

In the present study, a bacterial infection of cultured common dentex was described and proper antibiotics were determined. As *M. luteus* was previously used as a probiotic candidate, to determine the pathogenic status of this bacterium, histopathological changes of common dentex co-infected with *M. luteus* along with *Vibrio* sp. reared in a commercial fish farm located in the Aegean Sea region were described.

Material and Methods

Fish samples

In this study, 8 moribund common dentex (140-200g in weight) individuals were investigated. The moribund fish samples slowly swimming on the surface with various external disease symptoms were collected from the cages and autopsy was performed according to Roberts (2012).

Bacteriological examination and antimicrobial susceptibility testing

Bacterial inoculations were taken from the internal organs of fish samples and streaked onto Marine Agar 2216 and Tryptic Soy Agar (containing 1,5% NaCl) and incubated at 22ºC for 72h. After incubation, the isolated bacterial pure cultures were identified depending on their biochemical and phenotypic characters (Austin & Austin, 2016). For the determination of antimicrobial susceptibility of the isolates, a modified Kirby-Bauer disk diffusion test protocol was applied with three replicates (Bhunia et al., 1988). All isolates were tested for antimicrobial susceptibility against eight different antibiotic substances. Fresh cultures of isolates were streaked onto Mueller-Hinton agar (Oxoid), incubated at 22°C for 48h and results were interpreted based on the available CLSI (Clinic and Laboratory Standards Institute) data.

Histological examination

Histopathological tissue samples from the visceral organs and also gills and heart were taken and fixed in 10% buffered formalin solution and processed with routine methods (Roberts, 2012). Tissue samples were embedded in paraffin blocks, 5 µm sections were stained with hematoxylin-eosin (HE) and examined under light microscope equipped with a digital imaging software (NIS-Elements BR Microscope Imaging Software; Nikon Instruments).

Results

Moribund fish externally showed darkening of the skin, fin-base hemorrhages and loss of scales (Fig 1a). Internally, accumulation of a bloody fluid in the abdomen, hemorrhages on the liver, elongation in the spleen, liquefaction of the kidney and accumulation of a yellowish liquid in the intestine were observed (Figure 1b).

Gram-positive, non-motile cocci and Gram-negative motile bacils were recovered from the visceral organs of moribund fish samples. According to their morphological and biochemical characteristics given in the Table, Gram-positive cocci isolates were identified as *Micrococcus luteus* and Gram-negative bacils were identified as *Vibrio sp*.

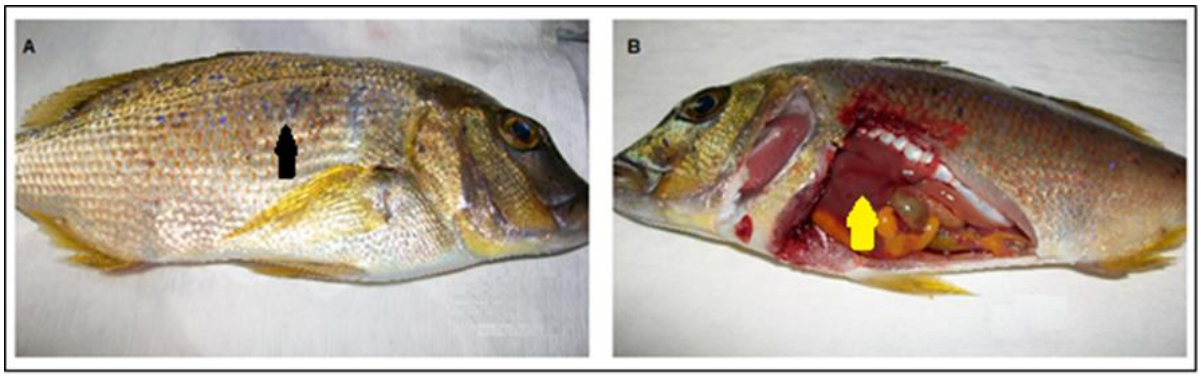

Figure 1: External and internal gross pathology of moribund dentex samples a) Loss of scales (arrowed) and darkening of the skin on the dorsal area b) Hemorrhages on the liver (arrowed), yellowish fluid accumulation in the intestine

In the antimicrobial susceptibility testing, *Vibrio* isolates were commonly determined to be sensitive against flumequine, oxytetracycline and kanamycin. *M. luteus* isolates were determined to be sensitive against same antibiotics besides erythromycin that the *Vibrio* isolates were determined to be resistant.

In the histopathological examination of infected fish tissues, various pathological changes were determined. In the histopathological examination of the infected fish tissues, an increase in the melanomacrophage centers in the spleen was observed (Fig. 2a). Degeneration and liquefactive necrosis in the spleen (Fig. 2a), heart muscle cells (Fig. 2b) and in the renal tubules of kidney (Fig. 2c) were detected. Edema in the tubule epithelium cells of kidney (Fig. 2c), melanomacrophage centers in kidney (Fig. 2c), hepatic lipidosis around the pancreas cells of liver (Fig. 2d) were seen. Infected gills showed hyperplasia in the gill lamellae and edema in the seconder gill lamellae (Fig. 2e). Also, enterocytes around the intestine lumen were necrotic and an abundance of the goblet cells were remarkable in proximal midgut of the intestines (Fig. 2f).

Table 1. Phenotypic and morhpological caracteristics of bacteria recovered from cultured common dentex

	Micrococcus luteus (*n:8*)	*Vibrio* sp. (*n:6*)
Colony colour	Yellowish	Cream
Morphology	Cocci in tetrads	Bacil
Gram Staining	+	-
Motility	-	+
Cytochrome Oxidase	+	+
Catalase	+	+
Coagulase	-	Not Tested
O/F	Oxidative	Fermentative
O/129 (150µg)	Resistant	Sensitive
Indole	-	+
Methyl Red	-	Variable
Voges-Prouskauer	Variable	Variable
Arginine	-	+
Lysine	-	-
Ornithine	-	-
ONPG	-	Variable
Inositol	-	-
Arabinose	-	-
Sorbitol	-	-
Production of H_2S	-	-
Citrate	Variable	Variable

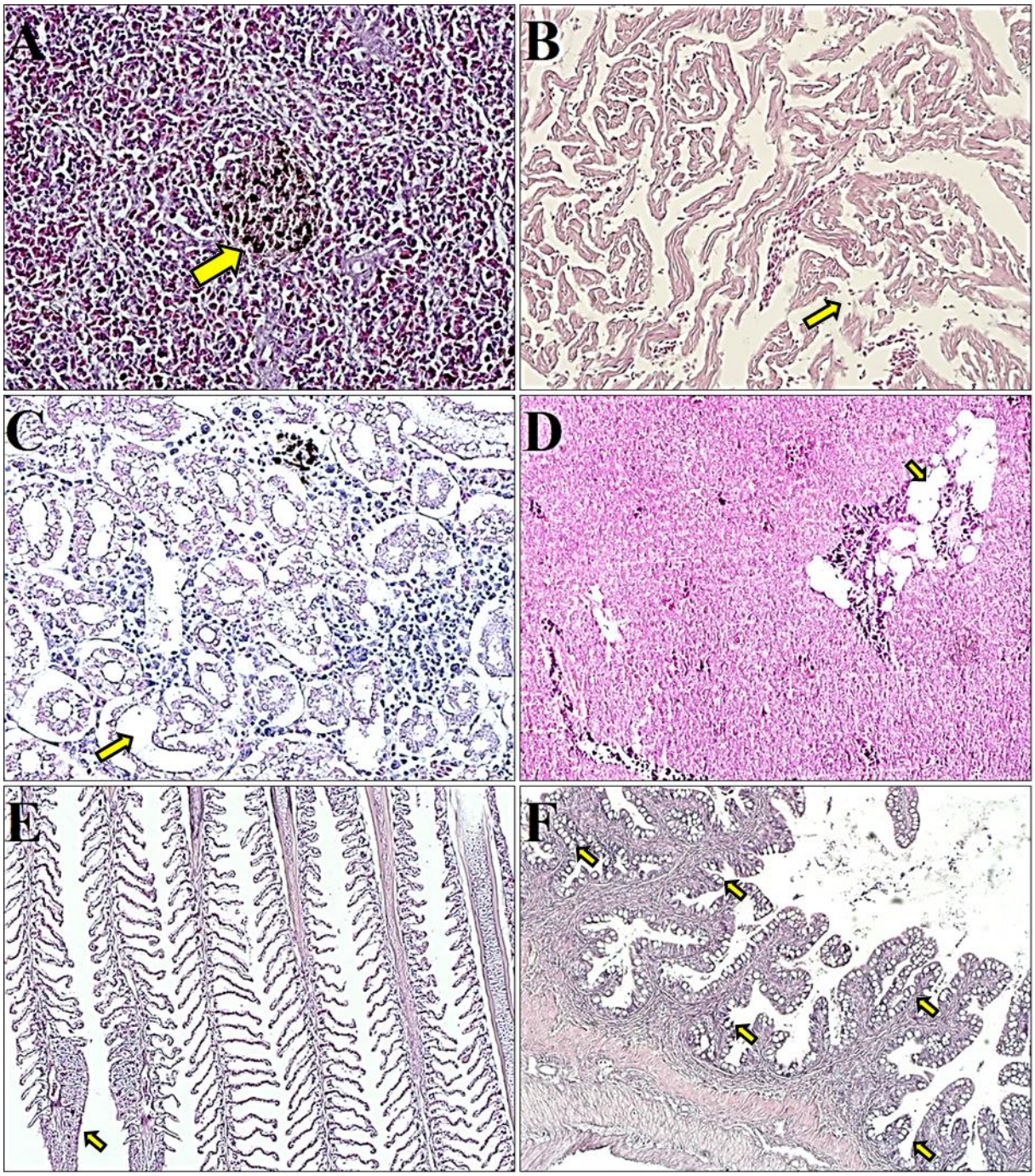

Figure 2: Histopathological changes in the fish tissues co-infected with *M. luteus* and *Vibrio sp*., stained with H&E. **a)** Melanomacrophage centers in the spleen (arrowed) (40X) **b)** Degeneration and necrosis in the heart (arrowed) (20X) **c)** Melanomacrophage centers, tubular degeneration (arrowed) and necrosis in the kidney (20X) **d)** Hepatic lipidosis in liver (arrowed) (10X) **e)** Hyperplasia in the gills (arrowed) (20X) **f)** Abundant goblet cells (arrowed) in proximal midgut of the intestines (10X)

Discussion

Common dentex is a fish species cultured in Turkey commercially in a small-scale production, bedsides a great amount of data on the pathologies of this species is needed for the success of mass production (Company et al., 1999; Saka et al., 2007, Rigos & Katharios, 2010). In this study, a mix infection case of cultured common dentex caused by *M. luteus* along with *Vibrio sp.* was reported, antimicrobials that can be used in the treatment of the disease were determined and the histopathological changes in the infected fish tissues were demonstrated firstly.

Vibriosis caused by the members of the Gram-negative bacterial genus *Vibrio*, is the most important disease of marine fishes, but recently this disease is reported in co-infection cases with other pathogens (Austin & Austin, 2016). Vibriosis is commonly

detected in cultured common dentex (Rigos & Katharios, 2010). *V. harveyi* is among the pathogens of major importance in dentex culture and previously reported from Spain (Company et al., 1999), Greece (Rigos & Katharios, 2010) and Turkey (Turgay & Karatas, 2016). Also recently a Gram-positive bacterium *Staphylococcus cohnii* subsp. *cohnii* co-infection case along with *Vibrio sp.* was reported in common dentex cultured in Turkey (Akaylı et al., 2011).

Micrococcus luteus, a Gram-positive bacterium was previously reported both as a pathogen of cultured rainbow trout (Austin & Stobie 1992, Aydın et al., 2005; Mousavi et al., 2010) and gilthead seabream (Çanak & Akaylı, 2018) and as a probiotic candidate. It's antagonistic effect against fish pathogenic *S. cohnii* subsp. *cohnii*, *S. aureus* and *S. epidermidis* isolates were revealed (Akaylı et al., 2016b).

Generally, infected fish samples showed darkening of the skin, finbase hemorrhages and loss of scales externally and, accumulation of a bloody fluid in the abdomen, hemorrhages on the liver, elongation in the spleen, liquefaction of the kidney and accumulation of a yellowish liquid in the intestine internally. These clinical symptoms showed similarities with the ones observed in *M. luteus* infections of rainbow trout (Austin & Stobie, 1992; Aydın et al., 2005; Türk et al., 2013), common dentex (Akaylı et al., 2015), gilthead seabream (Çanak & Akaylı, 2018) and sharpsnout seabream (Akaylı & Yardımcı, 2018). Also phenotypic and biochemical identification of the bacterial isolates were confirmed with the identification tables given by Austin & Austin (2016). However, various specific clinical symptoms such as skin hemorrhages and ulcers (Roberts, 2012; Austin & Austin, 2016) that are reported in vibriosis cases were not detected in the fish samples used in this study.

Histopathological changes of fish tissues infected with *M. luteus* was previously demonstrated in rainbow trout (Aydın et al., 2005). This is the first report on the histopathological changes of *M. luteus* infection in a marine fish species. As this bacterium caused general cell degenerations and necrosis in various tissues similar to those reported by Aydın et al. (2005), remarkable increase in the goblet cells of the intestines, hepatic lipidosis in the liver and hyperplasia in the gills were the main differences of the *M. luteus* infection of this fish species.

Hemorrhages and severe necrotic areas in various tissues such as liver, heart, kidney and spleen are among the typical histopathological changes in vibriosis cases (Roberts, 2012), but in this infection case, these changes were not observed in an advanced stage. Besides, the presence of melanomacrophage centers in various tissues were thought to be related with the secondary pathogen, *Vibrio sp.* recovered from the internal organs which is a general phenomenon caused by this bacterium (Roberts, 2012; Austin & Austin, 2016).

Fish intestine, an organ which plays an important role in specific and non-specific immune system contains goblet cells in the anterior part (Roberts, 2012). It was reported by Rinso et al. (2010) that goblet cells were normally filled when the intestine was exposed to bacteria. It was also detected that the last part of the midgut contains M-like cell which might play a role in mucosal immunity similar to goblet cells (Wallace et al., 2005). In this study, infected fish samples were detected to have goblet cells in the proximal midgut in an excessive number and this is thought to be related with the infection case.

In previous *M. luteus* infection cases, freshwater isolates were reported as sensitive to enrofloxacine, oxytetracycline, doxycycline and florphenicol (Aydın et al., 2005; Türk et al., 2013) while marine isolates were sensitive to erythromycin, oxytetracycline and florphenicol (Akaylı & Yardımcı, 2018; Çanak & Akaylı, 2018). Similarly, in this study, marine *M. luteus* isolates were sensitive to oxytetracycline and erythromycin. Also semi-resistance against enrofloxacin in marine isolates (Çanak & Akaylı, 2018) and resistance against flumequine was reported (Türk et al., 2013; Çanak & Akaylı, 2018). In contrast, our *M. luteus* isolates were sensitive against flumequine.

M. luteus is both found in the aquatic environment and in the gut flora of various fish species. Previously, antagonistic effect of *M. luteus* against common fish pathogens was reported. Also in some cases, especially related with the culture conditions and/or stress, it was recovered from moribund fish samples. Besides, a slight change in the antibacterial susceptibility profile of this species was detected in recent years. In conclusion, with this first report on the histopathological changes of *M. luteus* infection in a marine fish species, this bacterium was detected as a possible pathogen of common dentex with various damages. The pathogenic and probiotic status of this species should be considered and *in-vivo* pathogenicity assays should be carried out in freshwater and marine fishes.

Acknowledgement

This study was supported by the Istanbul University Research Projects Found with the project number 2637.

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