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Isolation and characterization of *Pseudomonas putida* caused granulomas in cultured sea bass (*Dicentrarchus labrax*) in Turkey

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ABSTRACT: The present study describes a *Pseudomonas* infection in diseased European sea bass (*Dicentrarchus labrax*) caused by *Pseudomonas putida* in Turkey. Detected symptoms in the diseased fish were internally white nodules in the liver and kidney. Bacteriological samples from the kidney, spleen, liver and blood were streaked onto Tryptic Soy Agar (TSA) and Brain Heart Infusion Agar (BHIA) containing 1.5 % NaCl. After incubation, bacterial colonies produced fluorescent pigment under the ultraviolet light were observed. The morphological and physiological characteristics of bacterial colonies were determined together with their biochemical characteristics by using API 20E and API 20NE, and isolated bacteria were identified as *Pseudomonas putida*. Furthermore, 16S rRNA gene of one isolate was partially sequenced and showed 99 % identity with the Genbank sequences of *P. putida*. Histopathologically, the granulomatous lesions and presence of Gram-negative basil shaped bacteria in these lesions were observed in the liver and kidney. This study represents the first report of *P. putida* isolation and identification as a primer agent and granulomas in the kidney and liver in the diseased sea bass in the Black Sea, Turkey.

Keywords: API 20E, API 20NE, cultured sea bass, granulomas, *Pseudomonas putida*, 16S rRNA gene

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INTRODUCTION

Fish pathogenic pseudomonads have been reported increasingly in the past decade. The causative agents of pseudomonas infection such as *Pseudomonas fluorescens* reported from rainbow trout (*Oncorhynchus mykiss*) (Akayli and Timur, 2004; Kaçar and Balta, 2017), Nile tilapia (*Oreochromis niloticus*) (Eissa et al., 2010), sea bream (*Sparus aurata*) (Turk, 2002) *P. luteola* from rainbow trout (Altınok et al., 2007), *P. plecoglossicida* from ayu, (*Plecoglossus altivelis*) (Kobayashi et al., 2000) and rainbow trout (Akayli et al., 2010), *Pseudomonas baetica* sp. nov. from wedge sole, (*Dicologlossa cuneate*) (Lopez et al., 2012), and *P. aureginosa* from Nile tilapia (*Oreochromis niloticus*) (Eissa et al., 2010).

Pseudomonas are ubiquitous inhabitants for oxygenated environments (Palleroni, 1984; Austin and Austin, 2016). *Pseudomonas putida*, a member of genetically related fluorescent pseudomonads, is an aerobic, Gram-negative basil shaped bacterium, and has been isolated and identified from diseased ayu (*Plecoglossus altivelis altivelis*) (Wakabayashi et al., 1996), yellowtail (*Seriola quinqueradiata*) (Kusuda and Toyoshima, 1976), European eel (*Anguilla anguilla*) (Fan, 2001), oyster toadfish (*Opsanus tau*) (Smolowitz et al., 1998), large yellow croaker (*Pseudosciaena crocea*) (Shen et al., 2008), black sea bream (*Sparus macrocephalus*) (Mao et al., 2010), and Nile tilapia (*Oreochromis niloticus*) (Eissa et al., 2010). Moreover, this bacterium is an opportunistic human pathogen responsible for bacteremia (Lombardi et al., 2002).

In Turkey, pseudomonas infection caused by *P. putida* was first diagnosed in scattered mirror carp (*Cyprinus carpio*), gold fish (*Carasius auratus*) (Aydın et al., 1998). After then it was reported from cultured rainbow trout (Altınok et al., 2006). *P. putida* has been not reported as a primer agent in any marine cultured or wild fish species in Turkey. This paper is the first report of *P. putida* infection in the diseased sea bass and histopathologically granulomas in the kidney and liver.

MATERIAL AND METHODS

Fish samples and clinical examination

Ten diseased sea bass (200-250 g) that showed rarely hemorrhagic ulcerative on the ventral body surface and generally white nodules on the liver and kidney were obtained from a floating marine cage farm located on the coast of the Black Sea in Turkey.

Bacteriological examination

Samples of kidney, liver, spleen, and blood were streaked onto Tryptic Soy Agar (TSA) and Brain Heart Infusion Agar (BHIA) supplemented with 1,5% NaCl. Plates were incubated at 20-21 °C for 24-48 hours. The isolates (n=10) recovered from sea bass were characterized by using conventional bacteriological method. In addition, these isolates were determined together with their biochemical characteristics using API 20NE and API 20E (Buller, 2004), however API suspension in 1.5% saline was used as inoculum for marine bacteria as described by Grisez et al., (1991).

Nucleic acid isolation and DNA sequencing

Total DNA extraction was performed with Roche Genomic DNA Purification Kit (11796828001, Germany) according to the manufacturer's instructions and used as template for PCR. An approximately 1400 bp long fragment of the 16S rRNA gene was amplified using the universal bacteria primer sets U8F (5' AGAGTTGATCATGGCTCAG 3') and 1492R (5'GGTTCACCTGTTACGACTT3') as reported by Weisburg et al (1991). PCR product were purified and sequenced by Medsantek (Istanbul, Turkey).

Antimicrobial susceptibility test

Antibacterial susceptibility of the isolates was determined by using KirbyBauer disc diffusion method according to Barry and Thornsberry (1985). For this purpose, 12 commercial antibiotic disc (chloramphenicol (30 µg/disc), kanamycin (30 µg/disc), flumequine (30 µg/disc), erythromycin (5 µg/disc), streptomycin (10 µg/disc), ciproflaxacin (1 µg/disc), sulphamethoxazole (25 µg/disc), ampicillin (10 µg/disc), enrofloxacin (5 µg/disc) florfenicol (30 µg/disc), oxytetracycline (30 µg/disc), furazolidon (50 µg/disc) (Oxoid, England)) were used. Results were carried out according to instruction of the Clinical and Laboratory Standart Institute (2008).

Histopathological examination

Tissue samples from the kidney, liver, spleen, heart, intestine, and gills were immediately fixed in 10% buffered formalin and processed for paraffin embedding. Histologic sections (4-5 µm) were stained with hematoxylin-eosin (H&E) and Brown-Hopps (B&H) and examined under light microscopy (Brown et al., 1973; Bullock, 1978).

RESULTS

Clinical findings

This outbreak occurred in April 2017 with cumulative mortality approximately 15 %. Water temperature was 15-16 C° at the time of sampling. While three sea bass samples externally showed hemorrhag-

ic ulcerative skin lesions on the ventral body surface (Figure 1a), seven fish appeared no external clinical sign on the body surface. Internally, teen sea bass generally exhibited pale liver, enlargement of the spleen, a clear fluid in the intestine and abdominal cavity, liquefaction of the head kidney and generally white nodules on the liver and kidney (Figure 1b).



Figure 1. Diseased sea bass. (a): Hemorrhagic ulcerative skin lesions (*) on the ventral body surface, around the pectoral and ventral fins; (b): White spot on the pale liver (*), and splenomegaly

Bacteriological and molecular identification findings

After the incubation of the bacteriological inoculations from the visceral organs and blood, pure and one dominant colony were isolated on TSA and BHIA (Figure 2a). The isolated bacteria (n=10) produced florescent pigment under the ultraviolet light (Figure 2b). They were motile, basil shaped, Gram-negative, cytochrome oxidase and catalase positive, and oxidative. Therefore, ten isolates were identified as *Pseudomonas* sp. According to the morphological, physiological and biochemical characteristics of the isolates, isolated bacteria were identified as *P. putida* (Table 1). Similarly, our isolates were characterized as *P. putida* by API 20 NE (profile number: 0140057) and API 20E (profile number: 220404643).

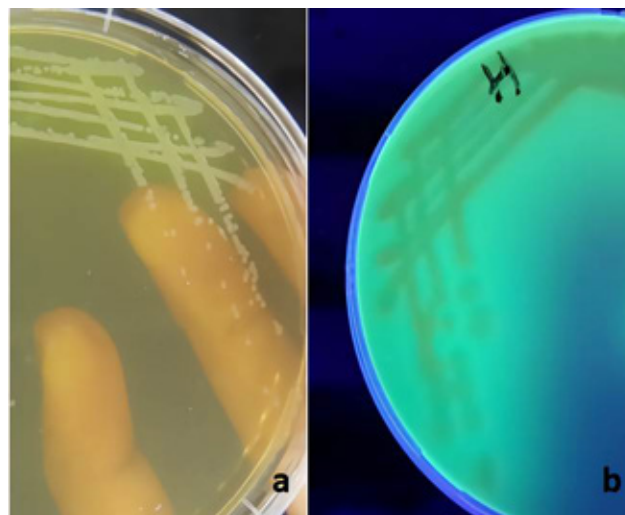


Figure 2. *P. putida* (a) produced fluorescent pigment (fluorescein) (b) under the UV light

Molecular identification findings

Gene sequencing with 16S rRNA revealed that the isolate was *P. putida* (99%). This result was derived from the National Center for Biotechnology Information (NCBI) blast database. The sequence obtained in this study is defined as GenBank accession number MH368654.

Antimicrobial susceptibility test findings

P. putida strains were found susceptible to all of the antimicrobials tested, except for oxytetracycline, erythromycin, streptomycin, kanamycin and ampicillin.

Histopathological findings

In the present study, histopathologically rarely melanomacrophage center, discharge of the white pulp and hyperemia in the spleen (Figure 3); epicarditis, the polymorph-nuclear leukocyte infiltration

and hypertrophy of the heart muscle cells (Figure 4); degeneration and necrosis of the epithelial cells of kidney tubules periglomerular and tubular oedema (Figure 5); vacuolar degeneration and karyolysis of hepatocyte, congestion (Figure 6a) hemorrhage among the hepatocyte cells (Figure 6b); hyperplasia of the gill filament (Figure 7) were observed. In addition, granulomas were noted in the kidney (Figure 8) and liver section (Figure 9a). Presence of Gram-negative bacteria were found within the necrotic granulomas (Figure 9b, Figure 10).

Table 1. Morphological, physiological and biochemical characteristics of the isolates recovered from diseased sea bass

| Characteristics | Isolates (n=10) | Characteristics | Isolates (n=10) |
|----------------------|-----------------|-----------------------------|-----------------|
| Gram staining | - | Growth at 4 C° | + |
| Motility | + | Growth at 41 C° | - |
| Cytochrome oxidase | + | β-galactosidase | - |
| Catalase | + | H ₂ S production | - |
| O/F | O | Arginine dihydrolase | + |
| Fluorescent pigments | + | Ornithine decarboxylase | - |
| O/129-10μg | - | Lysine decarboxylase | - |
| O/129-150 μg | - | Carbohydrate utilization | |
| Metil Red | - | Rhamnose | - |
| Voges Proskauer | - | Arabinose | + |
| Indole | - | Sucrose | - |
| Citrate | + | Mannitol | - |
| Nitrate | - | Maltose | - |
| Aesculine hydrolysis | - | Trehalose | - |
| Urease | - | Sorbitol | - |
| Starch hydrolysis | - | Melibiose | + |
| Gelatin hydrolysis | - | Inositol | - |
| Growth on MCA | + | Xylose | + |
| Growth on TSA | + | Lactose | - |

+: positive reaction, -: negative reaction, **O/F**: Oxidative/Fermentative, **MCA**: MacConkey Agar

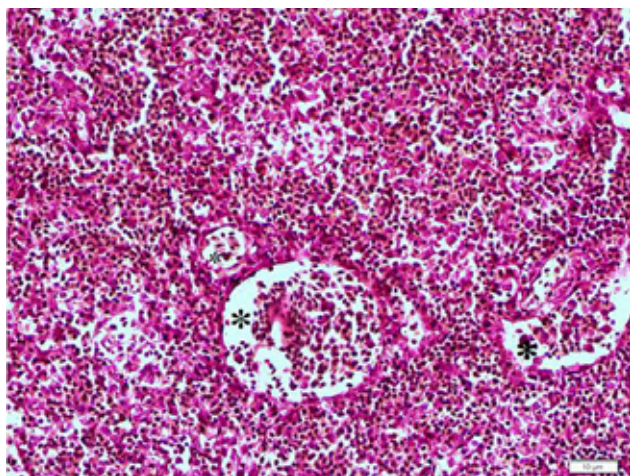


Figure 3. Discharge of the white pulp (arrowed), congestion (*) of spleen vessels (H&E) x1000

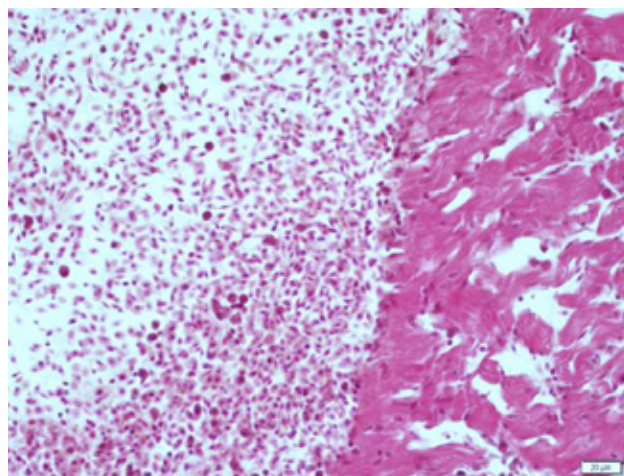


Figure 4. The polymorph-nuclear leukocyte infiltration (arrowed) and hypertrophy of the heart muscle cells (H&E) x400

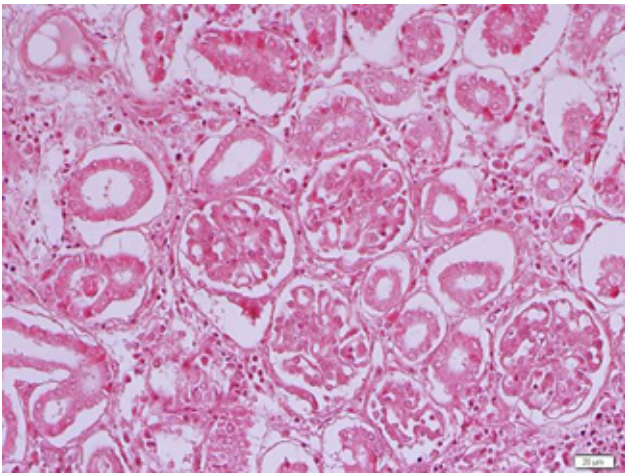


Figure 5. Periglomerular (arrowed), tubular oedema and tubular degeneration (H&E) x400

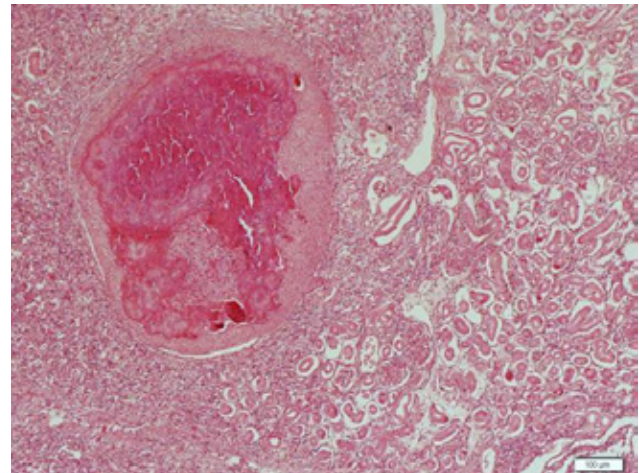


Figure 8. Granuloma in the interrenal haemopoietic tissue of kidney (H&E) X100

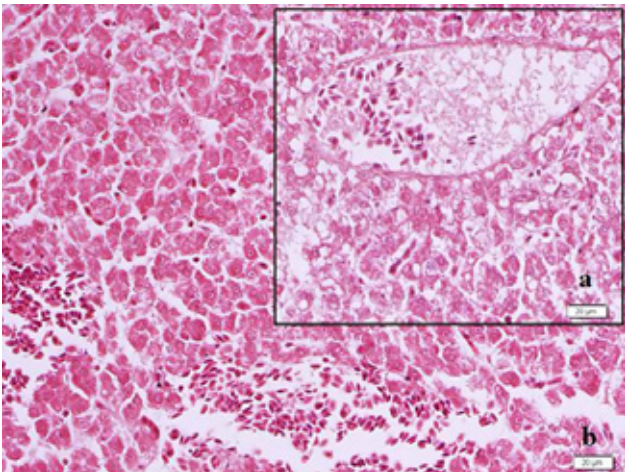


Figure 6. Congestion (a) and hemorrhage (b) among the hepatocyte (H&E) x400

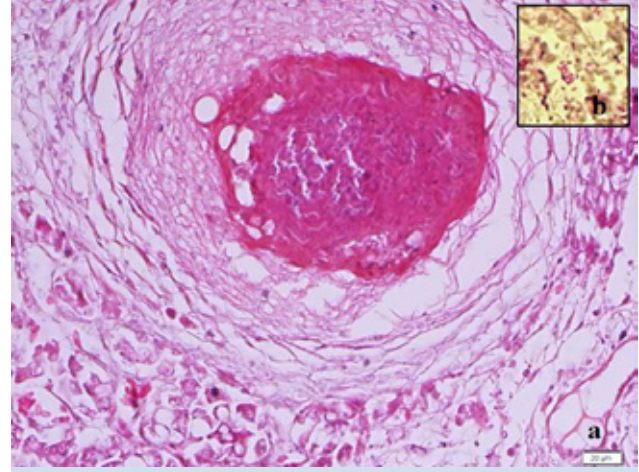


Figure 9. Necrotizing granuloma surrounded by epithelioid in the liver (a) (H&E), Gram negative bacteria in the granuloma (b) (B&H) X400

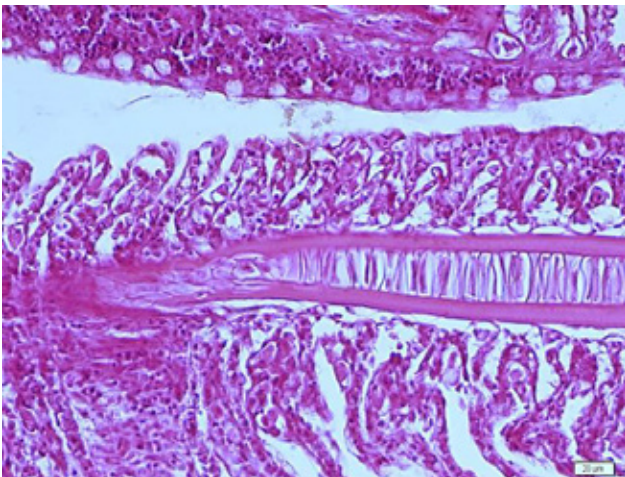


Figure 7. Gills showing hyperplasia of the respiratory epithelium and increased goblet cells (H&E) x400

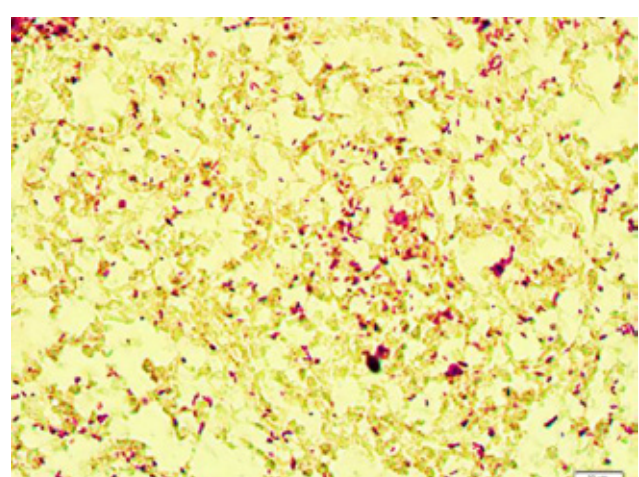


Figure 10. Presence of Gram-negative bacteria caused granuloma in the kidney tissue (B&H) X1000

DISCUSSION

Pseudomonas agents such as *P. fluorescens*, *P. putida*, *P. luteola*, *P. aeruginosa*, and *P. plecoglossicida* have increasingly been reported from the diseased cultured and wild fish species all over the world (Kobayashi et al., 2000; Akayli and Timur, 2004; Altinok et al., 2007; Eissa et al., 2010). However, there is no report about *Pseudomonas* infection caused by *P. putida* as a primer agent in the cultured marine fish species in Turkey.

In this study, diseased sea bass showed either external clinical signs or no external clinical signs as reported in the large yellow croaker (Shen et al., 2008). Although the ulcerative skin lesions on the base of the dorsal fin as reported in rainbow trout (Altinok et al., 2006) have not been observed in this study, three diseased sea bass showed externally ulcerative skin lesions around the pelvic and pectoral fins. Internally the gross pathology such as white nodules in the kidney and liver observed in the sea bass bear similarities to the large yellow croaker infected with *P. putida* (Shen et al., 2008). Similar gross pathological findings have been reported in large yellow croaker infected with *P. plecoglossicida* (Zhang et al., 2014). Altinok et al (2016) reported that there are no internal clinical findings in the rainbow trout. Therefore, it may be considered that the clinical signs of *Pseudomonas* infection caused by *P. putida* is not specific for different fish species.

In the present study, according to Gram staining, presence of cytochrome oxidase and catalase enzyme, oxidation, isolated bacteria were identified as *Pseudomonas* sp. presumptively *P. fluorescens* or *P. putida* for florescent pigment production under the ultraviolet light. The gelatin hydrolysis, which is the most important biochemical characteristics that distinguishes between these two fluorescent bacteria, has been defined as *P. putida* as it has a negative reaction. (Palleroni, 1984). Moreover, the morphological, physiological and biochemical characteristics of our isolates were very similar to those of *P. putida*, as described in previous reports (Palleroni, 1984; Buller, 2004; Austin and Austin, 2016). It has been reported sufficiency of rapid identification kits (API 20E, API NE) for the identification of *P. putida* isolated from diseased rainbow trout (Altinok et al., 2006). However, misidentification of the fish pathogens can occur when using of the API 20E (Kent, 1982; Topic Popovic et al., 2007). In present study, our isolates with API suspension in 1.5% saline were successfully

identified by API 20E and API 20 NE. In contrast to the results described by Altinok et al (2006) in current study we observed that the API 20 NE gave different profile number (0140057) compare with *P. putida* isolated from rainbow trout. Our isolates gave positive reactions at utilization of phenylacetic acid as distinct from rainbow trout isolates. For this reason, to identify and confirmation of *P. putida*, should be made rely heavily on biochemical tests.

In present study, it has been observed that *P. putida* strains were resistant to oxytetracycline, erythromycin, streptomycin, kanamycin, and ampicillin as described Altinok et al (2006). However, Kholil et al (2015) reported that oxytetracycline is highly effective against *Pseudomonas* species. In addition to this report, Saleh et al (2008) reported that *P. anguillaseptica* displayed sensitivity to erythromycin, oxytetracycline, and streptomycin as distinct from our results.

To date, there are no histopathological reports about infection of marine fish species with *P. putida*, except for freshwater fish species, rainbow trout. Altinok et al (2006) reported that histopathologically epithelial necrosis in the rainbow trout infected with *P. putida*, however in this study, the most prominent pathological changes were observed in the liver and kidney. The granulomas were observed in the kidney and liver as in the described large yellow croaker infected with *P. putida* and *P. plecoglossicida* (Shen et al., 2008; Zhang et al., 2014). Moreover, hyperemia in the spleen, epicarditis, degeneration and necrosis of kidney tubules, periglomerular and tubular oedema, hemorrhage vacuolar degeneration and karyolysis of hepatocyte, hyperplasia of the gill filament were observed in the diseased sea bass.

CONCLUSIONS

The pathogen causing *Pseudomonas* infection in the diseased sea bass was identified as *P. putida* for the first time. The gross and histopathological findings of the diseased sea bass indicated that *P. putida* is a potential pathogen of the cultured sea bass. This pathogen bacterium is important risk for the fish health so our further study will be experimentally carried out on pathogenesis and histopathology of *P. putida* infection in the cultured sea bass.

CONFLICT OF INTEREST

None declared by the author.

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This study was approved by the Istanbul University Local Committee on Animal Research Ethics (decision year 2013).

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