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Original Article

Fungal and Bacterial Co-Infection of Sea Bass (*Dicentrarchus labrax*, Linnaeus 1758) in a Recirculating Aquaculture System: *Saprolegnia parasitica* and *Aeromonas hydrophila*

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ABSTRACT

Aeromonas hydrophila causes symptoms of hemorrhagic septicemia in acute cases and can cause death in freshwater fish, whereas *Saprolegnia parasitica* is the cause of ulcers complicated by fungal mycelia located on skin that has lost its scales for various reasons. Both pathogens co-infect recirculating aquaculture systems at the İzmir Katip Çelebi University Fisheries Research and Training Center. Clinical, bacteriological, parasitological, and mycological studies were carried out on 25 fish samples during the infestation. Sabouraud glucose agar and malt extract agar were used to isolate the fungus, and the bacterial isolates were streaked on tryptic soy agar (Oxoid) with 5% defibrinated sheep blood. Gray-white cotton-like patches, erosions on the skin, and hemorrhaging were detected on the infected fish samples. The analytical profile index test and molecular identification showed that the bacterial agent was *A. hydrophila* and a fungal examination and amplification by polymerase chain reaction confirmed that the mycotic agent was *S. parasitica*.

Keywords: *Aeromonas hydrophila, Dicentrarchus labrax, Saprolegnia parasitica*

INTRODUCTION

Aeromonas hydrophila is an opportunistic microorganism that is widely distributed in water, soil and food (Laith and Najiah, 2013). It is a Gram-negative motile bacterium with aerobic and facultative anaerobic, oxidase-positive characteristics and can be found in aquatic environments and gastrointestinal tracts of healthy fish (Rey et al., 2009; Laith and Najiah, 2013). It is known as Bacterial Hemorrhagic Septicemia, Aeromonad Septicemia and Red Pest (Roberts and Shepherd, 2001) the major symptoms of which are skin ulcers, hemorrhaging and necrosis of visceral organs (Huizinga et al., 1979; Austin and Austin, 2007; Cipriano et al., 2001). A. hydrophila has a great impact on fish farms causing outbreaks with a mortality rate of 80-100% in a short period of time (Lukistyowati, 2012; Kusdarwati et al., 2017). To date the pathogen has been reported in various fish species such as mirror carp and gold fish (Timur, 1986), rainbow trout (Diler and Altun, 1995), guppy (Timur et al., 2003) and xiphophorus (Akayli and Zeybek, 2005) caught in Turkey.

Saprolegniasis is a mycotic freshwater disease that especially, affects fish and eggs (Gaikowski et al., 2003). It usually starts with cotton wool-like patches on the head and dorsal fin region then spreads all over the body as focal patches (Abdel-Aziz et al., 2002; Bangyakkum et al., 2003; Osman et al., 2008; Roberts, 2012). Erosion, poor water quality, overcrowding, handling, malnutrition, spawning or bacterial and parasitic infections may be the cause of this mycotic disease and it can lead to mortality (Noga, 1993; Pickering, 1994; Hussien et al., 2010). In Turkey, Saprolegniosis has, generally, been reported in freshwater fish species, especially in rainbow trout eggs (Diler, 1992).

Co-infections are described as infections caused by two or more genetically different pathogens and each pathogen has pathogenic

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effects that cause harm to the host when other pathogens are present (Cox, 2001; Bakaletz, 2004; Kotob et al., 2016). Interactions between different pathogens can have either synergistic or antagonistic effects on fish. Aeromonas hydrophila and Saprolegnia parasitica were detected in sea bass (*Dicentrarchus labrax*) with high mortalities and clinical signs of both pathogens in this study.

MATERIAL AND METHOD

Infected sea bass *(D.labrax*) with fungal infections and hemorrhages on different parts of the body were found in the university pond at İzmir Katip Çelebi University Fisheries Research and Training Center. Fish weighing approximately 40 gr were examined during the infestation and mortalities with water parameters were observed. 25 of the samples were subjected to clinical, microbiological, parasitological and pathological examination. An external and internal examination was carried out on the skin, abdomen, fins, gills, kidneys, intestines, liver and spleen. Bacterial examinations were conducted according to Austin and Austin (2007). Bacterial isolates were streaked on Tryptic Soy Agar (TSA, Oxoid; Merck, Germany) and Tryptic Soy Agar supplemented with 5% defibrinated sheep blood (BTSA) from the anterior of the kidneys, spleen and liver of the fish.

Fungal samples were provided from the patches on the body surfaces and streaked on Sabouraud Glucose Agar (SGA, Oxoid; Merck, Germany) and Malt Extract Agar (MEA, Oxoid; Merck, Germany). The bacterial samples were incubated at 21°C for 2 days and fungal samples were incubated at 21°C for 3-4 days. Native and colored samples were examined with a light microscope (BX53; Olympus, Japan) after incubation. Colorization of the fungus samples was conducted according to Arda (2006).

A scanning electron microscopic examination was carried out at İzmir Katip Çelebi University's Central Research Laboratory and for preparation, samples were sputtered with gold by QUORUM Q150 RES (Quorum Technologies, UK) and examined with a scanning electron microscope (Carl Zeiss 300VP, Germany).

Figure 1. Hemorrhagic septicemia and fungal growth on sea bass (*D.labrax*)

Aeromonas hydrophila strains were passaged onto TSA and BTSA three times in order to purify the colonies after primer isolation. They were then cultivated onto TSA for biochemical tests. Gram staining and an oxidase test were carried out according to standard procedures. The motility of the bacteria was detected by the hanging-drop technique. For further information, biochemical tests were carried out with API 20E (BioMerieux S.A., France) (Tanrıkul et al., 2004).

Molecular identification of the bacteria and fungus were conducted. The 16SrRNA gene sequence was polymeraze chain reaction (PCR) amplified in order to ensure that strains were *A.hydrophila.* Strains were obtained from the samples that were isolated from infected *D.labrax* during the outbreak. A EurX GeneMATRIX Tissue Bacteria DNA Isolation Kit (EURx, Poland) was used for DNA isolation. Then with a Thermo Scientific Nanodrop 2000 (ThermoFisher Scientific, USA), the density and quality of the isolates were determined. 27F and 1492R primers were used for PCR amplifications. Band screening of the PCR products was observed in the gel electrophoresis. Amplified products of template DNA were sent to the Macrogen direct sequencing service (Macrogen, Holland) for sequence determination. Sequences were thenchecked with the BLASTN 2.6.1. database. The Same procedure was used for fungus samples. DNA isolation was conducted using a EurX Tissue Bacteria DNA isolation kit. For PCR amplification, ITS1 and ITS4 primers were used and DNA samples were sent to Macrogen direct sequencing service (Macrogen, Holland) and sequences were checked with the BLASTN 2.6.1. database.

The antibiotic susceptibility of *A. hydrophila* strains was determined by the Kirby-Bauer disk diffusion method onto Mueller-Hinton medium (Hudzicki, 2009).

The water parameters such as temperature, salinity, oxygen and pH parameters were determined as 10°C, 3.93‰, 10.07 mg/L and 7.7, respectively. The suspended solid matter was analyzed with gravimetric methods (Stirling, 1985). Analyses of ammonium nitrogen, nitrite nitrogen, nitrate nitrogen, phosphate phosphorus, silica and chlorophyll-*a* were performed using a Spectrophotometer (DR 6000 Hach LANGE; Germany) (Strickl and Parsons, 1972; Parsons et. al., 1984; Stirling, 1985). Alkalinity, Ca and Mg were analyzed according to Egemen and Sunlu, (2003).

RESULT AND DISCUSSION

Stunned swimming, loss of balance and appetite were observed during the outbreak. Grey-white cotton like patches, erosion on skin and hemorrhages were shown on the infected fish samples. Mycelial growth and hemorrhages were observed on the fins and body surface (Figure 1).

Aeromonas hydrophila was isolated from the internal organs of sea bass with bacteriological studies from all the fish samples. Gram staining of the bacteria was shown in Figure 2. API test and molecular identification were also conducted (Figure 2, Table 1) with similar results.

The PCR amplification of the *A. hydrophila* gene sequence was registered in the BLASTN 2.6.1 database. It resulted in 100% nucleotide identity between the current isolate and *A. hydrophila* (accession number MF445123.1).

The antibiotic susceptibility test results are shown in Figure 3 and Table 2.

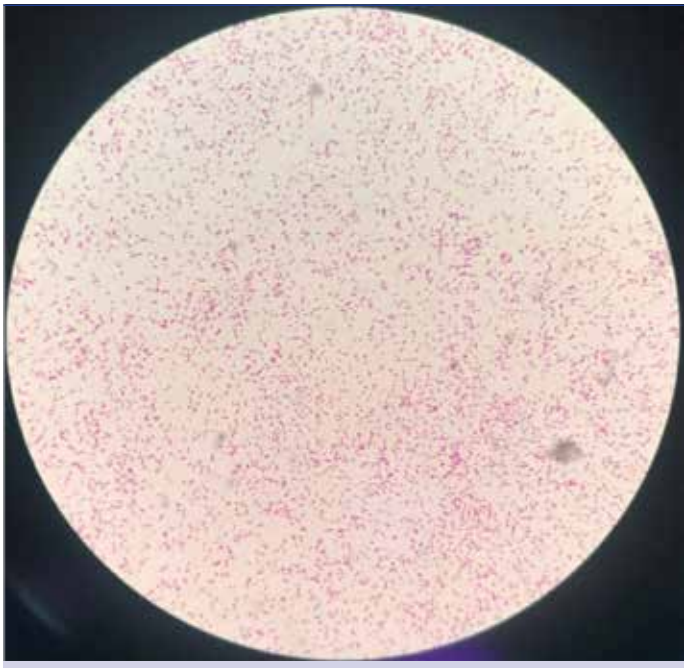

Figure 2. Gram staining of *A.hydrophila*

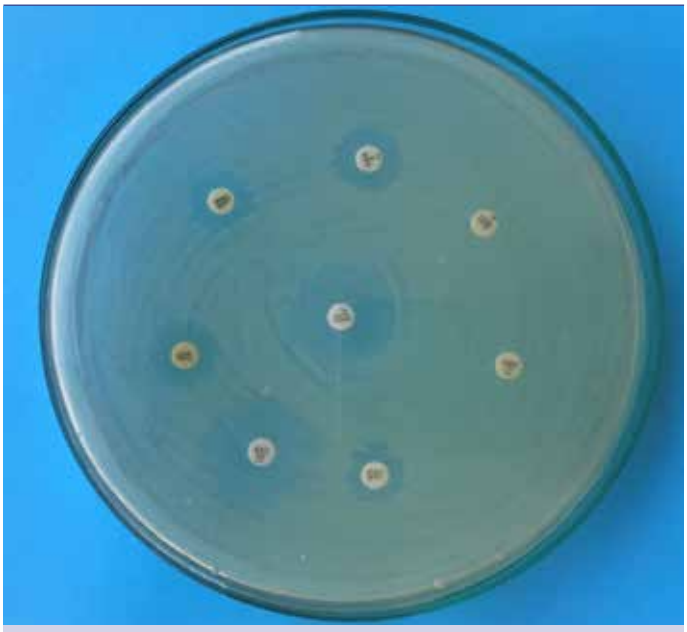

Figure 3. Antimicrobial susceptibility of *A.hydrophila*

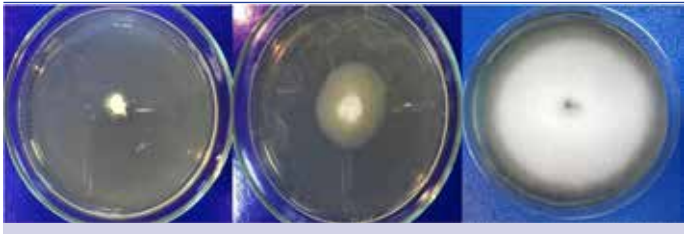

Figure 4. Fungal growth on SGA from skin samples of sea bass

Fungal growth was detected on SGA and MEA within 4 days of the samples being taken from mycelial growths on body surfaces (Figure 4). Long and branched hyphae with cysts were detected with a native and colored examination (Figure 5). Fungal zoospore was displayed from scanning electron microscopy overviews (Figure 6).

The PCR amplification of the 18S rRNA gene sequence was registered in the BLASTN 2.6.1 database. The FASTA homology showed 100% nucleotide identity between the current isolate and *Saprolegnia parasitica* (accession number AM228725.1).

Aeromonas hydrophila is a part of the normal intestinal microflora of healthy fish and stress is often a contributing factor (Yu et al., 2004). It is is an opportunistic pathogen (Arda et al., 2002) and effected by environmental factors such as oxygen, temperature, pH and stocking density (Klein and Wu, 1974). Like *A.hydrophila, Saprolegnia spp.* was reported from the natural environment of fish and, with suitable conditions, it is possible to be present as a secondary infection. Fish which are more vulnerable to physical conditions tend to be infected by Saprolegniosis (Neish and Huges, 1980; Diler, 1992). Saprolegniosis is considered as a secondary infection by Bruno et al. (2011) with immunosuppression, bacterial infections, poor husbandry and parasite infestations. The effect of this pathogen is reduced osmoregulation, which

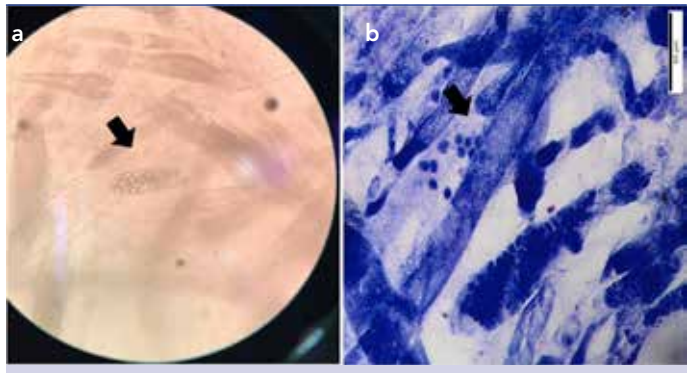

Figure 5. a, b. (a) Zoosporangiums of *S.parasitica* (x40) (b) Giemsa staining view of zoosporangiums

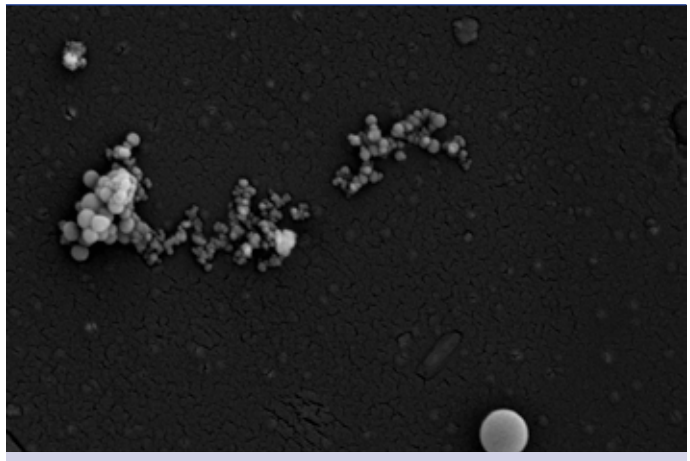

Figure 6. The zoospores of *S.parasitica*. SEM. Bar. 200 µm scale

Table 1. API 20E results of isolated bacteria confirmed that the isolate was *Aeromonas hydrophila*

ONPG	+	GLU	+
ADH	+	MAN	+
LDC	-	INO	-
ODC	-	SOR	+
CIT	-	RHA	-
H2S	+	SAC	+
URE	-	MEL	-
TDA	-	AMY	+
IND	+	ARA	+
VP	+	OX	+
GEL	+		

Table 2. The antibiotic susceptibility test results of *A. hydrophila*

Antibiotic (µg disc^{-1})	
Ampicillin (10)	R
Enrofloxacin (5)	I
Florfenicol (30)	I
Oxytetracycline (30)	I
Amoxycillin (10)	R
Doxycycline (30)	I
Sulphamethoxazole/Trimethoprim (25)	R
Flumequine (30)	I

R: Resistant, I: Intermediate, S: Sensitive

Table 3. Water parameters during the infestation

Temperature (°C)	10	NO_3^--N (mg/L)	0.101
Salinity (‰)	3.93	NO_3^- (mg/L)	0.461
Oxygen (mg/L)	10.07	PO_4^{-3}-P (mg/L)	4.16
pH	7.7	Chlorophyll-a(µg(L)	1.59
NH_4^+-N (mg/L)	0.136	Ca (mg/L)	280.54
NH_4^+ (mg/L)	0.175	Mg (mg/L)	272.23
NO_2--N (mg/L)	0.0115	Alkalinity ($CaCO_3$ mg/L)	338
NO_2^- (mg/L)	0.0385	Suspended solids	2.25
SiO_2 (mg/L)	6.45		

may even lead to death (Pickering and Willoughby, 1988). Kubilay et al. (2008) reported *Saprolegnia spp.* in rainbow trout fry infected with *Flavobacterium columnare* as a secondary opportunistic infection.

Aeromonas hydrophila has been reported in *Salmo gairdneri* (Peters et al., 1988), *Carassius auratus* (Citarasu et al., 2011), *Oreochromis niloticus* (Ibrahem et al., 2008), *Clarias batrachus* (Angka, 1990), *Cyprinus carpio* (Citarasu et al., 2011) with skin lesions and hemorrhagic septicemia over the body, tail and fin regions (Sarder et al., 2016). It is generally considered to be a secondary invader (Rogers, 1971) like Saprolegniosis. Clinical symptoms of both pathogens were detected on *D.labrax* in this study. Similarly, hemorrhagic septicemia, tail or fin rot, changes on the body surface, fins, gills and tail because of fungus infection were reported in catfish (*Clarias gariepinus*) with the same clinical symptoms as this study (Kusdarwati et al.,2017) caused by *A. hydrophila* and *Saprolegnia spp.*

The water parameters during the outbreak arre shown in Table 3. Temperature, salinity, oxygen and pH parameters were determined as 10°C, 3.93‰, 10.07 mg/L and 7.7, respectively. Suspended solid was measured as 2.25 mg/L while the Ministry of Food, Agriculture and Livestock of the Turkish Republic determined maximum 2 mg/L for sea bass farming.

Aeromonas hydrophila infections are mostly found in warm waters but under stress conditions, it may cause infectionsdown to 5°C. With a sudden increase of turbidity and a decrease in water temperature to 10°C, Saprolegniasis and *A.hydrophila* were observed together in this case.

The antibiotic susceptibility tests were conducted from isolated *A.hydrophila* strains but after applying 200 mL/tone formol bath for 30 minutes everyday and revision of physical conditions, it was no longer necessary to apply antibiotics to infected fish.

The problems in the filtration unit of İzmir Katip Çelebi University Fisheries Research and Training Center caused water pollution and mortalities were observed, particularly in tanks which had high stocking density. The mortality rate was calculated to be 18% during that period. The sudden change of water quality and intensive fish stock may have caused this agent to show itself as hemorrhagic septicemia in this study. The primer pathogen could not be determined but after fixing the filtration system and formol bath, the infections were successfully treated. This result suggested that *S.parasitica* could be the dominant pathogen in this case.

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