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THE FIRST PASTEURELLOSIS CASE IN CULTURED SEA BASS *(DICENTRARCHUS LABRAX* L.) AT LOW MARINE WATER TEMPERATURES IN TURKEY

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Abstract

The first observed pasteurellosis outbreak at a low marine water temperature (18-19ºC) is hereby reported. The disease was identified in three sea bass farms in the Aegean near Bodrum, Turkey. Diseased fish, *Dicentrarchus labrax* (L.), were characterized by lethargy, loss of appetite, darkened skin color, exophthalmia, abdominal swelling, pale gills, and hemorrhages over the operculum and ventral part of the body. Some diseased fish had hemorrhages on the head and opaque eyes. There were whitish nodules in the liver, the spleen varied in size (0.5-4 mm), and both organs were pale. Morphological, physiological, and conventional biochemical tests were used to determine the phenotypic properties of pure cultures of isolated colonies in samples taken from internal organs. Mono-Pp agglutination kit and API 20E were used to confirm the identified bacteria. The isolated strain was *Photobacterium damselae* subsp. *piscicida*. The sensitivity of the Aquarapid-Pp kit, which produced positive results in all diseased fish, was 0.91 and the specificity was 0.95. The principal histological changes were depletion of hemopoietic tissue and multiple round small or large necrotic and lytic areas in the spleen, vacuole degeneration and diffuse hemorrhagie in the liver, and peritubular vacuole degeneration and tubular necrosis in the kidney. Treatment with flumequine in the feed at 50 mg/kg body weight/day for seven days controlled mortality.

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Introduction

Pasteurellosis caused by *Photobacterium damselae* subsp. *piscicida* (*P. damselae* subsp. *piscicida*), formerly known as *Pasteurella piscicida*, is one of the most threatening diseases of natural and cultured marine fish (Magariños et al., 1996; Austin and Austin, 1999). It was first encountered in white perch *(Morone americanus)* and striped bass *(M. saxatilis)* in Chesapeake Bay (USA) in 1963, where it caused intensive deaths in natural populations (Sniezsko et al., 1964). The disease became important in Japan since 1969, principally affecting yellowtail *(Seriola quinqueradiata*; Kubota et al., 1970; Kimura and Kitao, 1971; Kusuda and Yamaoka, 1972; Egusa, 1983), black sea bream *(Acanthopagrus schlegeli*; Muroga et al., 1977; Ohnishi et al., 1982), and red sea bream (*Pagrus major*; Yasunaga et al., 1983). In Europe, this disease dates back to 1990, with incidents in gilthead sea bream *(Sparus aurata)*, European sea bass *(Dicentrarchus labrax)*, mullets *(Mugil cephalus* and *Chelon labrasus)* and sole *(Solea senegalensis)* in Spain (Toranzo et al., 1991; Balebona et al., 1992; Zorrilla et al., 1999), France (Baudin-Laurencin et al., 1991), Greece (Bakopoulos et al., 1995), Portugal (Baptista et al., 1996), and Turkey (Cagirgan, 1993; Candan et al., 1996; Timur et al., 1999; Tanrikul and Cagirgan, 2001). Serious economic losses result.

Pasteurellosis, a bacterial septicemia, is known as 'pseudotuberculosis' in Japan since white tubercles are seen in the spleen and kidney (Kubota et al., 1970). In the acute form of the disease, no intense findings are seen, but fish are lethargic and have hemorrhagic regions on the head and gills, and abnormal skin pigmentation (Austin and Austin, 1993; Magariños et al., 1996; Noga, 2000). The chronic form of the disease is characterized by white tubercles on visceral organs such as the spleen, liver, and kidney (Kubota et al., 1970; Magariños et al., 1996; Austin and Austin, 1999; Daly, 1999).

Pasteurellosis is closely related to increased water temperatures during the summer. Intensive mortality occurs in natural and cultured marine fish populations at water temperatures over 23ºC (Frerichs and Roberts, 1989; Kitao, 1993; Magariños et al., 1996). Candan et al. (1996) reported intense mortality from pasteurellosis in cultured sea bass, *D. labrax*, in a water temperature of 25ºC in Turkey. However, no reports seem to record incidents of pasteurellosis at low marine temperatures. This study describes the first observation of pasteurellosis at a low temperature of 18-19ºC.

Materials and Methods

An epizootic was recorded in November 2003 in three floating marine sea bass cage farms in the Aegean region of Bodrum where water salinity was 33‰ and temperature was 18-19ºC. Daily fish losses in the farms were 5%, 2.8%, and 4%, respectively. Fifteen fish (40-300 g) from each farm were selected and autopsied under sterile conditions at the fish disease laboratories of each farm. Samples were taken from visceral organs such as the spleen, liver, and kidney for bacterial isolations. Colonies were cultured on Brain Hearth Infusion Agar (BHIA, Merck) and Trypticase Soy Agar (TSA, Merck), supplemented with 2% NaCl. Cultures were incubated at 24ºC for 72 h.

Samples of kidney tissue (0.5±0.1 g) were immediately tested with the Aquarapid-Pp kit (*Photobacterium damselae* subsp. *piscicida* from Bionor AS, Skien, Norway, product code DN 240) according to the recommendations of the producer. The sensitivity and specificity of the test kit was evaluated according to the formulation reported by Rønning (1994). Standard petri and tube methods (Thornley, 1960; Toranzo et al., 1991; Thyssen et al., 1998) and API 20E (BioMerieux, France) were used to determine morphological, physiological, and biochemical properties. Mono-Pp agglutination kit (Bionor AS, Skien, Norway, DL 020) was used to confirm the identification of the bacterial strains isolated from the diseased fish.

The disk diffusion method was used to test the sensitivity of the isolates to antibiotics on Mueller-Hinton agar (Oxoid) by adding 1% NaCl (Bauer et al., 1966; Barry and

Thornsberry, 1985; NCCLS, 2003) to one of ten antimicrobial substances: flumequine (30 µg), penicillin G (10 µg), kanamycin (30 µg), ampicillin (10 µg), oxolinic acid (2 µg), tetracycline (30 µg), trimethoprim (5 µg), compound sulphonamide (300 µg), erythromycin (15 µg), and novobiocin (15 µg).

Samples for histological study were taken from muscle and gill tissues and the liver, spleen, kidney, stomach, and intestine. Samples were fixed in a 10% formalin solution and routinely processed to create 5 µm sections that were stained with hematoxylin and eosin and viewed under a microscope (Culling, 1963; Bullock, 1989).

A parasitological examination for crustaceans, protozoa, and monogeneans was performed on samples from the muscle, gills, liver, spleen, kidney, and air bladder (Collins, 1993).

Results and Discussion

The diseased fish were lethargic and had a loss of appetite, darkening of the skin, exophthalmia, abdominal swelling, pale gills, and hemorrhages over the operculum and ventral part of the body, notably in the pelvic and anal fins and around the anus. Whitish nodules of varying sizes (0.5-4 mm) were noted on the spleen, the liver and kidney were pale, and the gastrointestinal tract was empty. These results were similar to clinical and pathological findings reported by Balebona et al. (1992), Cagirgan (1993), Candan et al. (1996), and Zorrilla et al. (1999). Some fish had notable ocular lesions of exophthalmia and opacity, and white nodules in the liver. No ecto and/or endoparasites were found.

After incubation, the bacterial strains isolated from moribund fish (n = 15) produced convex (1-2 mm diameter), bright, grayish-yellow uniform colonies that were Gram-negative; positive for bipolar stainable rods, nonmotile cocobacilles, cytochrome oxidase, and catalase; fermentative; and sensitive to vibriostatic agent O/129 (10 and 150 µg/disk; Table 1). TCBS (Thiosulphate-Citrate-Bile salt-Sucrose agar, Merck) and MacConkey agar (No. 3: Oxoid) showed no bacterial growth. The isolated strains (n = 10) had similar phenotypic properties to other *P. damselae* subsp. *piscicida* strains (Toranzo et al., 1991; Thyssen et al., 1998; Austin and Austin, 1999; Daly, 1999; Zorrilla et al., 1999), showing that our strains belonged to the same subspecies.

Aquarapid kits reduce the diagnosis and identification times of bacterial pathogens and their resultant diseases. The Aquarapid-Pp kit can be useful in early diagnosis of pasteurellosis outbreaks in hatcheries and growout facilities (Magariños et al., 1996). In field studies, Aquarapid-Pp kit results gave positive results in all diseased fish. The sensitivity and specificity of the kit was 0.91 and 0.95, respectively. Therefore, as Rønning (1994) and Romalde et al. (1995a) suggested, the Aquarapid-Pp kit was able to identify *P. damselae* subsp. *piscicida*.

The Mono-Pp kit caused significant agglutination in the *P. damselae* subsp. *piscicida* strains while the control reactant did not. No cross-reaction occurred when the Mono-Pp kit was applied to other members of the *Vibrionaceae* family such as *P. damselae* subsp. *damselae*, *Listonella* (*Vibrio*) *anguillarum* serotype 02, *V. ordalii*, and *V. harveyi*, in agreement with the findings of Romalde et al. (1995b), Candan et al. (1996), and Magariños et al. (1996). This result was quite useful for confirming the identification of the isolate.

Identification was also confirmed by the API 20E system. Isolated *P. damselae* subsp. *piscicida* strains from the moribund fish had a profile of 2 005 004.

The *P. damselae* subsp. *piscicida* strains were sensitive to chemicals generally used in pasteurellosis treatment such as ampicillin, flumequine, novobiocin, oxolinic acid, and tetracycline (Toranzo et al., 1991; Magariños et al., 1996; Balebona et al., 1998; Zorrilla et al., 1999; Thyssen and Ollevier, 2001). Toranzo et al. (1991), Bakopoulos et al. (1995), Balebona et al. (1998), and Thyssen and Ollevier (2001) reported that the *P. damselae* subsp. *piscicida* strains they studied were resistant to kanamycin. However, all strains in our study were sensitive to kanamycin. The flumequine treatment effectively controlled mortality at the farms.

Table 1. Results of standard physiological, biochemical, and API 20E tests of bacteria isolated from moribund sea bass.

Test	*Standard method*	*API 20E*
Motility	-	NA
Gram stain	-	NA
Bipolar stain	+	NA
Oxidase	+	+
Catalase	+	NA
O/F (Leifson)	Fermentative	NA
Indol production	-	-
Voges-Proskauer	(+)	+
Metil-Red	+	NA
ADH	+	+
LDC	-	-
ODC	-	-
H_2S production	-	-
Gelatinase	-	-
Urease	-	-
Growth at:		
4ºC	-	NA
22ºC	+	NA
37ºC	-	NA
Growth in:		
0% NaCl	-	NA
3% NaCl	+	NA
6% NaCl	-	NA
Gas from glucose	-	NA
Acid production from:		
Amygdalin	NA	-
Arabinose	-	-
Glucose	+	+
Inositol	-	-
Lactose	-	NA
Mannitol	-	-
Mellibiose	NA	-
Rhamnose	NA	-
Sorbitol	NA	-

Table 1 cont'd

Sucrose	-	-
Citrate utilization	-	-
NO_2 production	-	-
ONPG (β-galactosidase)	-	-
Hemolysis of erythrocytes:		
Sheep	-	NA
Growth on:		
MacConkey agar	-	
TCBS	-	
Resistance to:		
0/129 (10 μg/disk)	Sensitive	
0/129 (150 μg/disk)	Sensitive	
		Inhibition Zone Diameter (mm)
Ampicillin	Sensitive	25
C. sulphonamides	Resistant	-
Erythromycin	Resistant	10
Flumequine	Sensitive	40
Kanamycin	Sensitive	15
Novobiocin	Sensitive	38
Oxolinic acid	Sensitive	30
Penicillin G	Sensitive	40
Tetracycline	Sensitive	30
Trimethoprim	Sensitive	40

+: positive; -: negative; (+): weak positive
NA: not applied

The diseased fish had depleted hemopoietic tissue and multiple round small or large necrotic areas in the spleen, either with or without a few cellular inflammatory cells (Figs. 1, 2). There were vacuole degeneration, diffuse hemorrhagie, and necrotic areas with chronic inflammatory cells in the fatty liver tissue (Figs. 3, 4). In the kidney, there were peritubular vacuole degeneration, tubular necroses, and considerably reduced renal interstitium, while the remaining cells were necrotic (Fig. 5). Histological changes were similar to those reported by Toranzo et al. (1991), Timur et al. (1999), and Noga (2000) for pasteurellosis. In addition, there were hemorrhagie and hyperplasia on the base parts of the secondary lamellae of gill filaments. However, no changes were detected in intestinal mucous membrane and muscular tissues.

Pasteurellosis occurs in cases of low marine water quality, high temperature (over 23ºC), and high salinity (20-30‰; Toranzo et al., 1991; Cagirgan, 1993; Candan et al., 1996; Magariños et al., 1996; Noga, 2000). In this study, pasteurellosis was observed at a

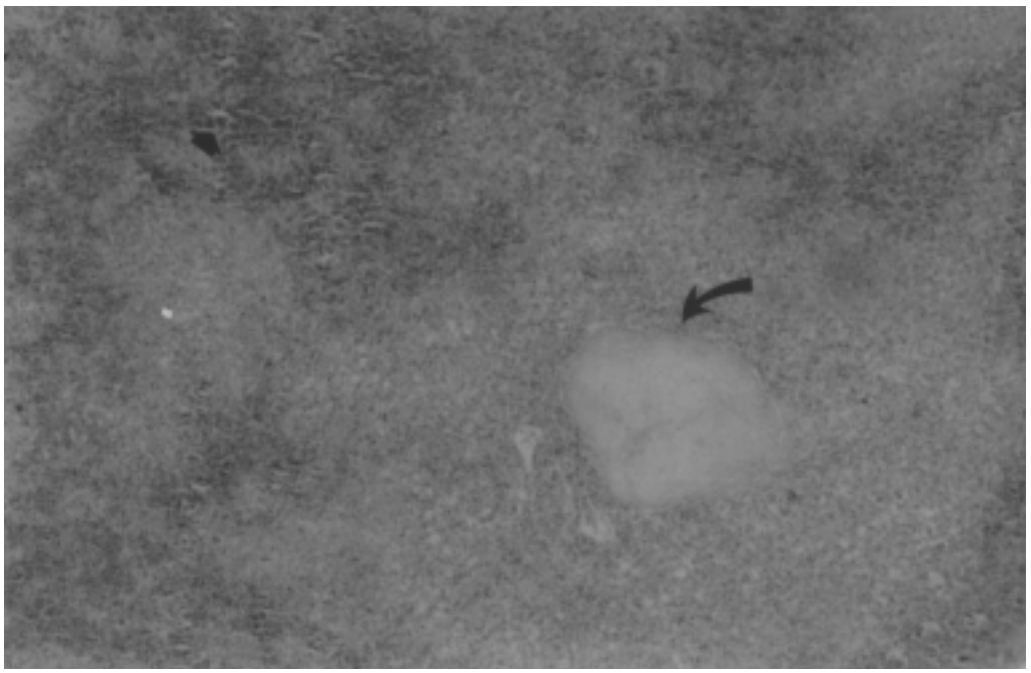

Fig. 1. Depletion of hemopoietic tissue (short arrow) and multiple round necrotic and lytic area (long arrow) in the spleen, H + E x 250.

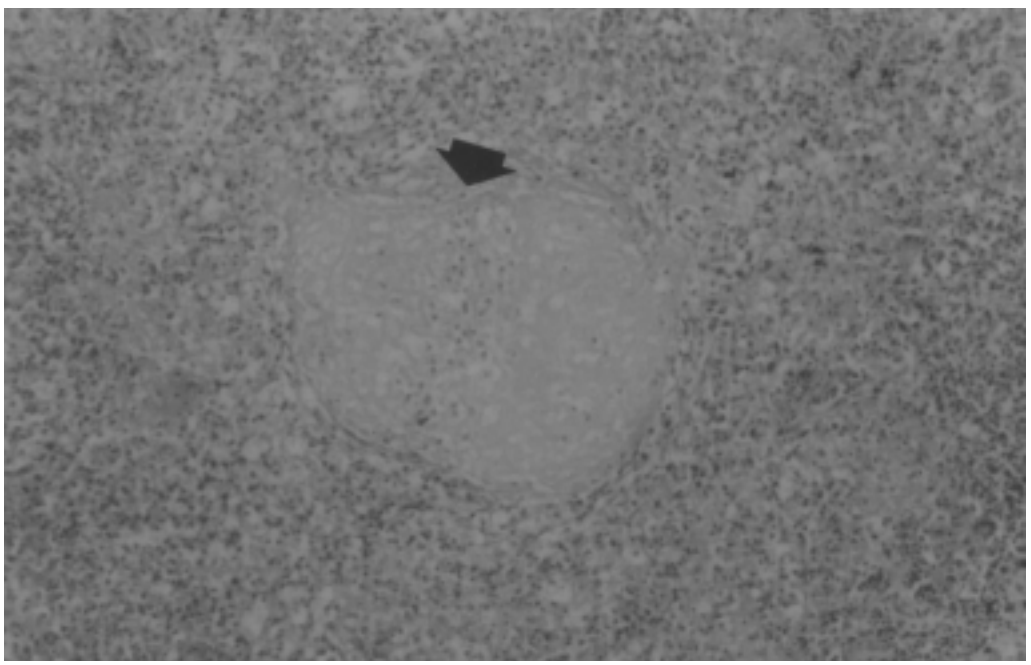

Fig. 2. Encapsulated necrotic nodule (arrow) in the spleen, H + E x 500.

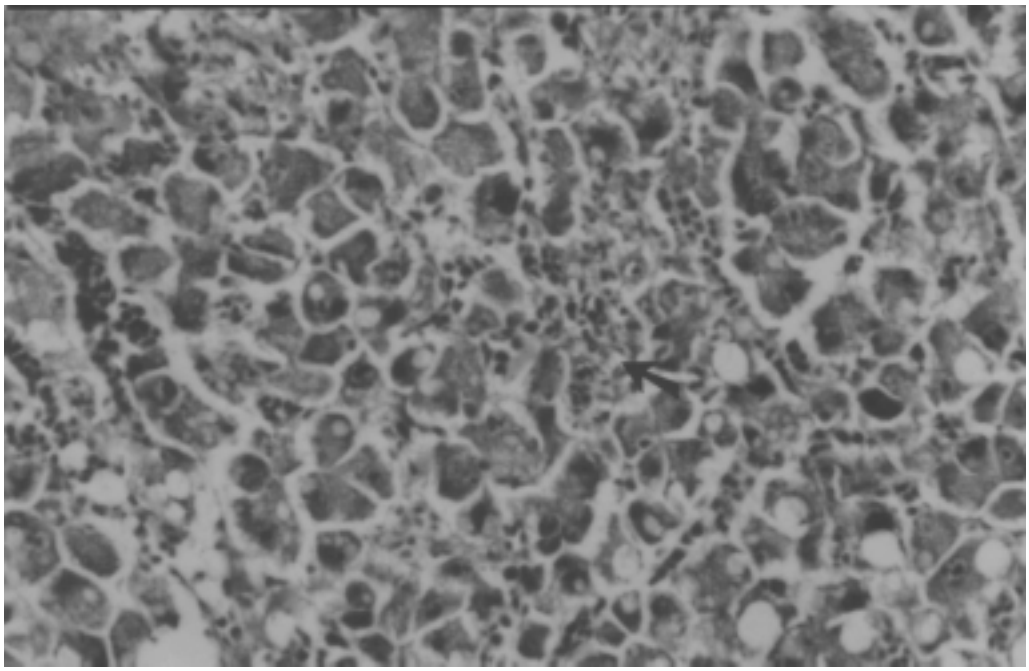

Fig. 3. Vacuole degeneration and hemorrhagie (arrow) in the fatty liver, H + E x 500.

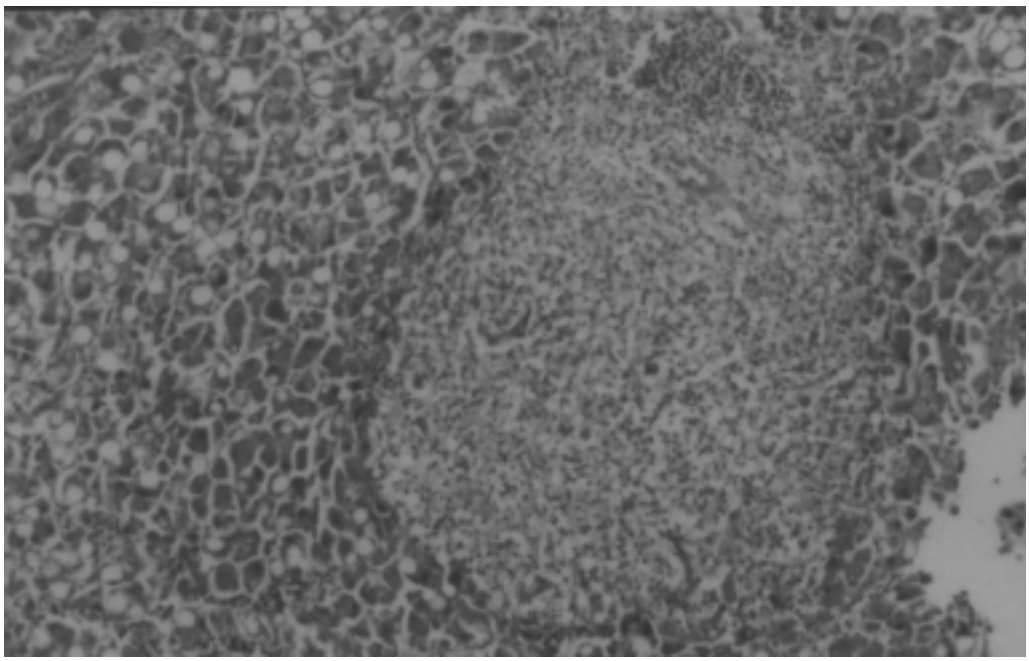

Fig. 4. Necrotic area with chronic inflammatory cells in the hemorrhagic fatty liver, H + E x 500.

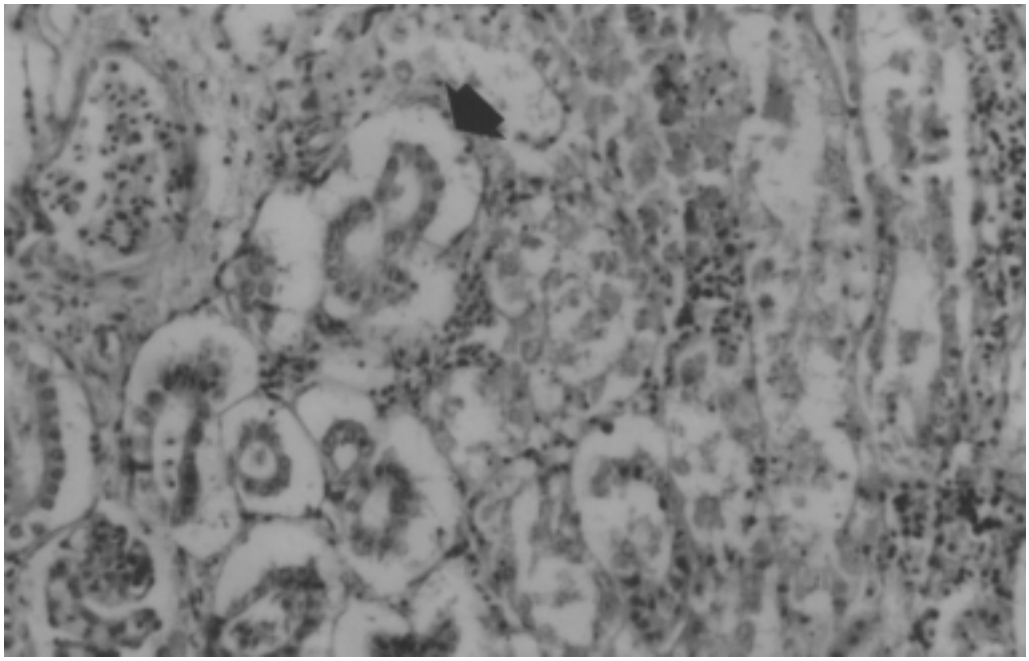

Fig. 5. Peritubular vacuole degeneration (arrow), tubular necrosis, reduced renal interstitium, and necrotic cells in the kidney, H + E x 500.

low marine water temperature of 18-19ºC for the first time. The outbreaks occurred in November 2003, causing daily mortality of 2.8-5% and considerable economic losses. This indicates that the disease has become endemic to sea bass farms in the Aegean.

In conclusion, the first incident of pasteurellosis in cultured sea bass, *D. labrax*, at a low marine water temperature of 18-19ºC is reported. The etiological agent was identified as *P. damselae* subsp. *piscicida* using morphological, physiological, and conventional biochemical tests, the Aquarapid-Pp rapid diagnostic kit, the Mono-Pp agglutination test, and histopathological study.

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