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Occurrence of *Photobacterium damselae* Subsp. *Piscicida* in Sea-Cage Farmed Meagre (*Argyrosomus regius*) in Tenerife, Canary Islands, Spain

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Abstract During 2008, the bacterium *Photobacterium damselae* subsp. *piscicida* was identified in meagre (*Argyrosomus regius)* culture during three disease outbreaks which occurred in Tenerife, Canary Islands, with mortalities reaching 50%. Typical disease signs were observed, including the characteristic spleen nodules, and bacterial identity was confirmed by biochemical and serological tests. This is the first reported isolation of *Photobacterium damselae* subspecies *piscicida* associated with mortalities in meagre.

Keywords Bacteria · Pathogen · Meagre · Aquaculture · Atlantic Ocean · *Photobacterium damselae* subspecies *piscicida*

Meagre (*Argyrosomus regius*) is a new species for aquaculture in the southern Mediterranean and, in less than 10 years, has become the third most produced marine species in this area (FEAP 2012). The emergence of meagre as an important species in Mediterranean aquaculture is because of their high growth rates, and the ability to exploit existing production technologies already used for seabass (*Dicentrarchus labrax*) and gilthead seabream (*Sparus aurata*), which simplifies diversification of farm production. Meagre can grow to 50 kg, with a supreme flesh quality (*regius* for royal quality of flesh) (Poli et al. 2003).

The presence of pathogens may be one of the major constraints for the development of new species in aquaculture (Rigos and Katharios 2010). Reports on diseases affecting meagre have been scarce and mainly focused on the identification of parasites such as the nematode *Philometra* sp. (Moravec et al. 2007), monogeneans *Benedenia sciaenae* (Toksen et al. 2007), *Sciaenocotyle pancerii* (Merella et al. 2009; Quilichini et al. 2009; Ternego et al. 2010), *Calceostoma* sp. (Duncan et al. 2008) and the dinoflagellate *Amyloodinium ocellatum* (Soares et al. 2012). In 2006, an outbreak of *Photobacterium damselae* subsp. *damselae* was detected in southern Spain (Labella et al. 2011). Also, the presence of two genotypes of betanodavirus (VNN, Viral Nervous Necrosis) was detected in wild meagre caught on the Atlantic coast of the Iberian Peninsula (Lopez-Jimena et al. 2010).

Photobacterium damselae subsp. *piscicida* (*Phdp*) is one of the most significant bacterial pathogens in the Mediterranean, reaching this area and the Canary Islands in the 1990s and causing significant mortality in farmed gilthead seabream and seabass, and in new cultured species such as *Solea senegalensis* or *Seriola dumerilli* (Baudin-Laurencin et al. 1991; Toranzo et al. 1991; Balebona et al. 1992; Sarasquete et al. 1993; Baptista et al. 1996; Real et al. 1997).

In August and September 2008, juvenile meagre were brought to Tenerife from hatcheries in France. Transport to the island was performed in specially adapted trucks, travelling by road to Barcelona and then by sea to Tenerife. During transfer, the fish were held at a density of 51–54 kg m^{-3} and oxygen levels were maintained above 12–13 mg L^{-1} O_2. During the boat trip, water exchanges were performed to maintain water quality. All shipments of fish were examined on arrival in Tenerife, using the procedure described below, in

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Table 1 Mortality and isolation of *Photobacterium damselae* subsp. *piscicida* (*Phdp*) associated with each outbreak (A, B and C)

Outbreak	A	B	C
Mortality associated with outbreak (% of stock)	25	50	30
Total number of fish sampled	10	10	10
Photobacterium damselae subsp. *piscicida* (*Phdp*) isolated from (% of sample)	70	100	60
Phdp present as pure culture from (% of sample)			
Kidney	40	100	30
Spleen	60	100	60
Liver	40	100	30
Phdp present in mixed culture from (% of sample)			
Kidney	30		30
Spleen	10		
Liver	30		30

order to assess the heath status. Most of the fish examined exhibited minor erosion of the tail fin, but no parasites or bacterial growths were detected.

Within a month of stocking in Tenerife, increased mortality occurred in meagre on a number of different farm sites, located in two of the main aquaculture areas. Sampling was not conducted further because of the aquaculture company closure. Seven of these cases were investigated to determine the cause of mortality. In each case, ten representative moribund fish were euthanized by severing the spinal cord and samples were taken for analysis. Parasitological examination was performed by microscopic observation of gills, bile and intestine. Samples from kidney, liver and spleen tissues were taken for bacteriological analysis and were inoculated onto Tryptic Soya Agar (1.5% NaCl, TSA, Oxoid) and Thiosulphate Citrate Bile Salts Sucrose (TCBS, Oxoid) and incubated at 22 °C for up to 7 days. Where a bacterial isolate was obtained as pure culture from all the organs, a representative colony from spleen culture was used for further characterisation and identification. When a mixed culture was obtained, one isolate of each colony type was used for further analysis and identification. Isolates were identified based on morphological, phenotypical and biochemical characteristics such as: Gram staining, bacterial morphology, bipolar staining, motility, presence of cytochrome oxidase, catalase, arginine dihydrolase, lysine and ornithine decarboxylases (Moeller Decarboxylase Broth,

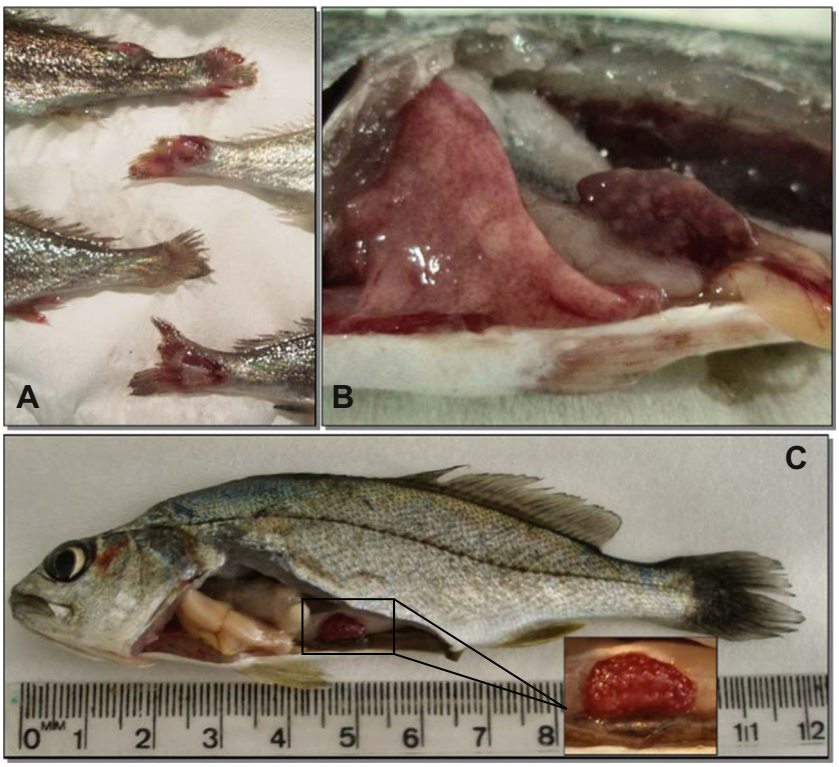

Fig. 1 **a** - Erosion of tail and fin; **b** - Hemorrhagic liver and hypertrophic spleen with whitish granulomatous nodules; **c** - Hypertrophic spleen with whitish granulomatous nodules of *P. damselae* subsp. *piscicida*

Table 2 Isolation of other bacteria than *Photobacterium damselae* subsp. *piscicida* (*Phdp*) from outbreaks A and C

Outbreak	A	C
Other bacterial species isolated from (% of sample)		
Pseudomonas sp.	30	
Vibrio harveyi (Identax score - 97.8%)	10	
Vibrio mediterranei (Identax score - 97.6%)		30
Vibrio alginolyticus (Identax score - 98.3%)		20
Pseudomonas sp. (% of sample)		
Kidney	30	
Spleen	10	
Liver	20	
Vibrio harveyi (% of sample)		
Kidney	10	
Spleen		
Liver	10	
Vibrio mediterranei (% of sample)		
Kidney		30
Spleen		
Liver		20
Vibrio alginolyticus (% of sample)		
Kidney		20
Spleen		
Liver		10

Oxoid), oxidative-fermentative metabolism (OF acc. Hugh and Leifson, Fluka), utilisation of citrate (Simmons Citrate Agar, Fluka), production of indole and nitrate reduction, urease, gas production from D-glucose, sensitivity to Vibrostat O/129 and growth in the presence of NaCl (0%, 3%, and 10%). All media were supplemented to reach a 1.5% NaCl content. The use of the dichotomous key by Toranzo and Barja (1995) permitted to perform a presumptive bacteria identification. In order to identify the *Vibrio* species, individual supplementary tests were performed and identification was made to species level according to Noguerola and Blanch (2008). Once the tests for *Vibrio* identification were completed, results were analysed using the open software IDENTAX (www.identax.org) (Flores et al. 2009) in order to obtain a score for the success of identification. Identification was performed using software default settings with variable test results ranging between 15%–85% and with a successful identification dependent on a score > 95%. The presumptive diagnosis of *Phdp* was made using the biochemical tests above according to Magariños et al. (1992, 1996) and Thyssen et al. (1998, 2005), and by absence of growth on TCBS agar (Rajan et al. 2003). Due to the high serological homogeneity of the *Phdp* (Romalde 2002) confirmation of the *Phdp* identity was made using the Bionor™ Mono-Pp specific agglutination test (Romalde et al. 1995; Buller 2004).

Phdp was detected in three of the seven cases investigated. The onset of mortality in these 3 cases occurred 15, 18 and 29 days after the fish were stocked in sea cages (referred to as outbreaks A, B and C respectively). Data relating to each outbreak are given in Table 1.

The mortality registered was almost 50% in outbreak B and 20–30% in the other two outbreaks (Table 1). The mean weight of the 10 fish sampled from each outbreak was: outbreak A, 7.2 g (5.4–8.2 g); outbreak B, 13.2 g (11.1–14.9 g); outbreak C, 15 g (11–21.4 g). Of the 30 fish sampled, 10 were below the expected weight. Severe erosion of the tail fin was evident in 23 fish, but no other external lesions were observed

Table 3 Presumptive identification tests with used with Toranzo and Barja (1995) dichotomous key

	Photobacterium damselae subsp. *piscicida*	*Vibrio spp.*	*Pseudomonas spp.*
Gram Stain	–	–	–
Short bacilli	+	+	+
Motility	–	+	+
Oxidase	+	+	+
Nitrate reduction	–		
Oxidation-fermentation (O/F) (glucose)	F	F	O or NR
Gelatinase	–		
Gas production from Glucose	–		
Resistance to:			
O/129	–	+	+
Ampicillin (10 μg)		+	
Grown:			
0% NaCl	–	–	
22 °C		+	
Tryptic Soy Agar + 2% NaCl		+	

F fermentative, *O* oxidative, *NR* non reactive

Table 4 Phenotypical characterisation of *Photobacterium damselae* subspecie *piscicida*, *Vibrio harvey, Vibrio mediterranei* and *Vibrio alginolyticus*

	Photobacterium damselae* subspecie *piscicida	***Vibrio harveyi***	***Vibrio mediterranei***	***Vibrio alginolyticus***
Gram Stain	−	−	−	−
Morphology	Bacilli	Bacilli	Bacilli	Bacilli
Bipolar stain	+	−	−	−
Motility	−	+	+	+
Arginine dihydrolase	+	−	−	−
*Ly*sine decarboxylase	−	+	−	+
Ornithine decarboxylases	−	+	−	−
Catalase	+	+	+	+
Citrate production	−	−	−	−
D-glucosamine cs		−		
Gas from Glucose	−	−	−	−
Gelatinase	−	−	−	+
H_2S production	−	−	−	−
Indol production	−	+	+	+
Methyl red	+	+	−	+
Nitrate reduction	−	+	+	+
Oxidase	+	+	+	+
Oxidation-fermentation (O/F) (glucose)	F - NG	F - NG	F - NG	F - NG
Urease	−	−	−	+
Voges-Proskauer	+	−	−	−
β-galactosidase (ONPG)	−	−	−	−
Resistance to:				
O/129 10 μg	−	−	−	+
O/129150 μg	−	−	−	−
Novobiocin	−			
Ampicillin (10 μg)		+	+	+
Grown in:				
0% NaCl	−	−	−	−
3% NaCl	+	+	+	+
5% NaCl (*Phdp)*; 6% NaCl (*Vibrio)*	−	+	+	+
10% NaCl	−	+	−	−
Grown at:				
4 °C (*Vibrio*); 5 °C (*Phdp)*	−	−	−	−
25 °C	+	+	+	+
37 °C (*Phdp)*; 40 °C (*Vibrio)*	−	−	−	+
Grow on:				
Tryptic Soy Agar +2% NaCl	+	+	+	+
Marine agar	+	+	+	+
TCBS	−	Y	Y	Y
Acid production from:				
Amylase	−	+	−	+
Arabinose	−	−	+	+
Frutose	+			
Galactose	+			
Glucose	+	+	+	+
Mannitol	−	+	+	+

Table 4 (continued)

	Photobacterium damselae* subspecie *piscicida	***Vibrio harveyi***	***Vibrio mediterranei***	***Vibrio alginolyticus***
Mannose	+	+	+	+
Melibiose	−	−	+	−
Sucrose	−	+	+	+

Bold and italic: Tests used by Noguerola and Blanch (2008);

F fermentative, *F – NG* fermentative no gas formation, *Y* yellow

(Fig. 1a). Internally, the abdominal cavity appeared normal in only 4 of the samples, with splenomegaly and kidney enlargement recorded in 18 and 13 of the 30 fish sampled, respectively. Haemorrhaging of spleen, kidney and liver was also observed (Fig. 1b). The typical "pasteurellosis spleen" with whitish granulomatous nodules (Fig. 1b and c) was observed in 4 fish of outbreak B.

The presumptive bacteria identification cluster all the bacteria into three groups: Phdp, *Vibrio* spp. and *Pseudomonas*. *Phdp* was isolated from 23 of the 30 fish sampled. *Phdp* was isolated in pure culture from kidney, spleen and liver of all 10 fish sampled from outbreak B, in 4 of 10 fish from outbreak A and 3 fish from outbreak C. *Phdp* was also isolated as abundant pure growth from the spleen of an additional 2 fish from outbreak A and 3 fish from outbreak C. It was also isolated as the principal organism in mixed cultures from spleen, kidney and liver of some of the remaining fish (Table 1).

In outbreaks A and C, *Vibrio* spp. and a *Pseudomonas* sp. were isolated from some of the fish, as part of the mixed culture (Table 2) although *Phdp* was the most predominant colony type in all cases. The bacteria phenotypic characteristics are described in Tables 3 and 4.

Outbreak C was preceded by another outbreak 8 days after the fish were transferred to the sea cages, in which *V. harveyi* was also identified (Identax score of 99.6%).

No parasites were detected in the gills, intestine or bile of any of the fish analysed.

Since the first observation in Chesapeake Bay (Snieszko et al. 1964), outbreaks of pasteurellosis have been reported all over the world (Romalde 2002). The pathogen is considered ubiquitous in the Canary Islands having been isolated from all seabass and seabream farm sites in Tenerife and most of the site locations in the archipelago (J. Perez, *pers. comm.*). All outbreaks in meagre occurred within a month from fish arrival in Tenerife. While efforts are made to ensure optimal transport conditions, transport to the island takes almost one week. During this period fish are not fed and it may take up to one week following transfer for normal feeding behaviour to resume. This extended transfer time prolongs the period of potentially stressful conditions under which B-cell and T-cell functions may be affected, reducing fish resistance to opportunistic pathogens (Bonga 1997). In addition, water temperatures of 21–22 °C were registered at farms sites in Tenerife, which is above the permissive temperature (20 °C) for *Phdp* infections (Magariños et al. 2001). Affected meagre weighted less than 22 g at the time of the outbreaks. An age/size dependency for pasteurellosis outbreaks has been reported in other fish species such as *Sparus aurata*, with animals over 50 g being resistant to infection (Toranzo et al. 2005). Taking into account all these factors, it appears that the disease outbreaks in meagre associated with *Phdp* resulted from multiple factors including transport stress, introduction to a new environment, permissive water temperatures (> 20 °C) and fish age/size which rendered the fish susceptible to infection by the pathogen.

The recovery of other bacterial species in two of the three cases, and the disease outbreak associated with *V. harveyi* do indicate a susceptibility to bacterial infection. The presence and identification of several taxa is a common factor when dealing with farm samples, and Austin and Austin (2007) suggested that this may result from synergistic interaction between the taxa. However, the recovery of *Phdp* as the sole isolate in 17 of 30 fish sampled and its predominance in samples with more than one taxon, suggests that it is the main infection and that its presence might have "helped" the infection success of the other taxa.

However, *Phdp* is a known pathogen and all of the new species in Mediterranean aquaculture such as *Seriola dumerilii*, *Pagrus auriga, Dentex dentex* and *Solea senegalensis* are susceptible to this bacterium (Crespo et al. 1994; Labella et al. 2006; Rigos et al. 1998; Zorrilla et al. 1999). The high mortality registered (50%) in an outbreak where *Phdp* was the sole pathogen identified and the relatively high mortality (20–30%) registered in other two outbreaks where it was present at a high prevalence in a mixed infection, suggests the involvement of this bacterium with the registered mortalities.

Commercial vaccines have been effective in the prevention of pasteurellosis in other fish species such as gilthead seabream and seabass, although only one vaccine has been successful in juvenile stages (Toranzo et al. 2009). However, outbreaks as a result of co-infection of *Phdp* and *Vibrio* species are recurrent in fish farms, which led to the development of divalent vaccines, such as the one developed for *S. senegalensis* by Arijo et al. (2005). Experimental infections with *PhdP* should be performed in order to elucidate the real

virulence of this bacterium to meagre and the immune response that can be elicited. These studies are fundamental for developing vaccines and early vaccination programmes against *Phdp* and *Vibrio* for this species, in order to safeguard the promising future for meagre culture in the Mediterranean.

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