First observation of rickettsia-like organisms in cultured sea bass (Dicentrarchus labrax) in Turkey

G. Timur¹*, M. Timur¹, T. Akayli¹, J. Korun² and K.D. Thompson³

¹Department of Aquaculture, Fisheries Faculty, University of Istanbul, 34470, Laleli Istanbul, Turkey; ²Department of Aquaculture, Fisheries Faculty, University of Akdeniz, Kampus, 07049, Antalya, Turkey; ³Institute of Aquaculture, Stirling University, Stirling, FK9 4LA, UK.

Abstract
This paper describes the first observation of rickettsia-like organisms (RLOs) in cultured European sea bass (Dicentrarchus labrax) in Turkey. Generally, fish infected by the organism were anorexic, lethargic and experienced high levels of mortality during disease outbreaks. Internally, most of the affected fish exhibited grey to yellow coloured multifocal nodules in their kidneys and spleens. Although Listonella (Vibrio) anguillarum and Photobacterium damselae subsp. piscicida were isolated from diseased fish, clinical and pathological examination also indicated the presence of RLOs in diseased sea bass. Histopathologically, multifocal necrosis of parenchyma cells of the spleen, liver, kidney skeletal and heart muscle was observed, associated with an inflammatory reaction. Diseased fish also contained what appeared to be RLOs within the cytoplasmic vacuoles of their blood monocytes. The presence of RLOs in tissues of diseased fish was confirmed by immunohistochemistry (IHC).

Introduction
Rickettsia-like organisms (RLOs) have been reported as a major pathogen of fish. Infection by these organisms has been observed in farmed coho salmon (Oncorhynchus kisutch) in Chile since 1981, and since this time infection by rickettsia has caused major losses to the salmon farming industry in Chile (Cvitanich et al., 1991; Branson and Nieto Diaz-Munoz, 1991; Turnbull, 1993). RLO’s have since been reported in five species of cultured tilapia in Taiwan (Chern and Chao 1994; Chen et al., 1994; Fryer and Mauel, 1997), farmed Atlantic salmon (Salmo salar) in Chile, Norway, Canada and Ireland (Rodger and Drian, 1993; Alday-Sanz et al., 1994; Lannan at al., 1999; OIE, 2003), blue eyed-plecostomus (Panaque suttoni) in North Carolina, USA (Khoo et al., 1995), grouper (Epinephelus melanonstigma) in Taiwan (Chen et al., 2000) and white seabass (Atractoscion nobilis) in California, USA (Chen et al., 2000a). In France, Comp et al., (1996) found RLOs in moribund juvenile European sea bass (Dicentrarchus labrax) exhibiting abnormal swimming associated with nervous tissue necrosis. Both antigenic similarities (Steiropoulos et al. 2002) and the relatedness of these organisms to Piscirickettsia salmonis, the rickettsial agent that affects salmon, have been confirmed (McCarthy et al., 2005).

* Corresponding author’s email: gulsentimur@yahoo.com
Farming of sea bass in floating net cages on the coast of the Black Sea in Turkey started in 2001. In June 2003, heavy mortalities (30 %) occurred in sea bass reared in floating cages in this region at three fish farms located closely together. Clinical and pathological examination indicated that RLOs were present in diseased sea bass. This paper describes these findings.

Materials and methods

Fish
Twenty-five moribund fish (90-250 g), from three farms located on the coast of the Black Sea in Turkey, were sampled for histopathology and bacteriology. Affected fish were observed swimming near the surface or edges of the cages at the time of sampling. Water temperature at this time varied from between 20 and 22 °C.

Bacteriology
Samples of kidney, liver and spleen were streaked onto Tryptic Soy Agar (Merck, Darmstadt, Germany) supplemented with 1.5 % NaCl and TCBS agar (Merck, Darmstadt, Germany), and plates were incubated at 22°C for 24 h. The morphological and physiological characteristics of a representative number of bacterial colonies from each plate were determined (Austin and Austin, 1987; Austin and Austin, 1999), together with their biochemical characteristics using API 20E and API 50CH, and bacteria identified following Bergey’s Manual (Holt et al., 1994).

Histology
Samples of tissues from gut, kidney, liver, spleen, heart, gills and skeletal muscle were processed for histopathology by fixing in 10 % buffered formalin, and processed for paraffin embedding. Histological sections (5 µm) were stained with haematoxylin and eosin, Giemsa and Gram stain and examined by light microscopy (Bullock, 1978). Blood smears were prepared from blood sampled from the caudal peduncle collected into tubes containing anticoagulant (EDTA 4 mg/ml³) (Bullock, 1978), and stained with Giemsa (Amlacher, 1970).

Immunohistochemistry (IHC)
Immunohistochemistry was carried out as described by McCarthy et al., (2005). Anti-Piscirickettsia salmonis polyclonal antibody (kindly provided by the late Professor J. L. Fryer, Dept. Microbiology, Oregon State University, Corvallis, USA), diluted 1/100 in
Figure 2. Histological examination of diseased sea bass (a) Focal necrosis parenchyma cells, accompanied by a chronic inflammatory infiltration by mononuclear cells (arrowed) in the kidney. (H&E x50); (b) Multifocal necrosis of parenchyma cells in the spleen, accompanied by a chronic infiltration of inflammatory cells (arrowed) (H&E x50); (c) Focal necrosis parenchyma cells in liver, accompanied by a chronic infiltration of mononuclear cells (arrowed) (H&E x250); (d) Pericarditis, endocarditis (arrowed) and necrosis of myocardial fibres with accompanying chronic inflammation (H&E x250); (e) Focal necrosis of skeletal muscle fibres, accompanied by chronic infiltration of inflammatory cells (arrowed) (H&E x125); (f) Mild glomerular nephritis with increased mononuclear cell infiltration in the kidney (H&E x500); (g) Numerous enlarged vacuolated inflammatory cells containing rickettsia like organisms present as basophilic granules in the kidney (H&E x125).
Tris-buffered saline (0.2 M TBS, pH 7.2) followed by a goat anti-rabbit IgG horseradish peroxidase (HRP) conjugate (Diagnostics Scotland, Edinburgh, UK), diluted 1/50 in TBS was used to stain RLOs present in the tissue sections, while a monoclonal antibody against *Photobacterium damselae* subsp. *piscicida* (Ph. d. subsp. p)(Aquatic Diagnostics Ltd., Institute of Aquaculture, University of Stirling, Scotland) followed by a goat anti-mouse IgG HRP conjugate (Diagnostics Scotland), diluted 1/50 in TBS was used to stain *Ph. d. subsp. p*. The reaction was developed using a VIP staining system (Vector, Peterborough, UK) following the manufacturers instructions. Slides were mounted in Pertex and examined under a light microscope.

**Results**

Affected fish, weighing between 90 to 250 g, generally showed darkening of the skin, pale gills, lethargy, anorexia and significant levels of mortalities in fish stocks (up to 30 %). The fish were seen swimming near the surface or edges of the cages and had lesions on their skin which ranged from small patches to shallow haemorrhagic ulcers (Figure 1a) or orbital and abdominal dropsy.

Internally, the most obvious lesions observed were enlarged spleens and swollen kidneys with grey to yellow multifocal nodules (Figure 1b). The most prominent histological changes were seen in kidney, spleen, liver, heart and skeletal muscle. Multifocal necrosis of parenchyma cells, accompanied by a chronic inflammatory infiltration of mononuclear cells was observed in the kidney (Figure 2a), spleen (Figure 2b), liver (Figure 2c), heart (Figure 2d) and skeletal muscle (Figure 2e). Necrotic thrombi were present in some blood vessels, often with necrotic changes in the endothelium of vessels in the kidney and spleen. The liver sections displayed focal to diffuse necrosis of hepatocytes with mononuclear cell infiltration in the liver parenchyma (Figure 2c), vascular and perivascular necrosis, and intravascular coagulation resulting in fibrin thrombi within vessels. All three organs had numerous enlarged vacuolated cells containing RLOs as basophilic granules (Figure 2g). Pericarditis, endocarditis and necrosis of myocardial fibres were observed with accompanying chronic inflammation (Figure 2d). Necrosis and chronic inflammatory cell infiltration were found in sections of skeletal muscle (Figure 2e). Necrosis and hyperplasia of the epithelium resulted in lamellar fusion in the gills. Chronic inflammatory cell infiltration was found in the lamina propria and submucosal layer of the stomach and intestine.

![Figure 3](https://example.com/figure3.png)

**Figure 3.** Giemsa stained blood smears from affected fish (a) a few small, coccoid, basophilic rickettsia-like organisms within enlarged monocytes. (mag x1667); (b) Rickettsia-like organisms free in the blood, most of them showing binary fission. (mag x1667).
and sloughing of the epithelial cells of the gut mucosa was also seen.

The most striking change noted in the peripheral blood of infected fish was the large number of large monocytes present. Most of these cells contained small, coccoid, basophilic RLOs within cytoplasmic vacuoles (Figure 3a). RLOs were also observed free in the blood, many of which were undergoing binary fission (Figure 3b).

Mixed infections were observed in many of the sampled fish with pathogenic Gram negative bacteria cultured from visceral organs following incubation of kidney, spleen and liver swabs at 22 °C for 24 h. These bacteria were identified as either *Listonella* (*Vibrio*) *anguillarum* or *P. d.* subsp. *p.*

The presence of RLOs (Figure 4a) and *P. d.* subsp. *p.* (Figure 4b) in infected tissue sections was confirmed by IHC in association with the lesions.

**Discussion**

Since the recognition of RLOs in coho salmon in 1989 in Chile, the presence of rickettsial pathogens in fish has become increasingly apparent (Fryer and Mauel, 1997; Turnbull, 1993; Fryer and Hedrick, 2003). The incidence and associated mortalities due to rickettsiosis have increased dramatically (Fryer and Hedrick, 2003). Until 1994, all recognised rickettsial disease of fish had been observed in salmonids cultured in sea water (Fryer and Mauel, 1997). However, rickettsial disease has since been introduced into freshwater salmon (Fryer and Hedrick, 2003), several species of tilapia in both marine and freshwater ponds in Taiwan (Chern and Chao 1994; Chen et al., 1994; Fryer and Mauel, 1997), in blue-eyed plecostomus in USA (Khoo et al., 1995); grouper in Taiwan (Chen et al., 2000), white seabass (*Atractoscion nobilis*) in California, USA (Chen et al., 2000a) and European sea bass in France (Comps et al., 1996).

The clinical signs, gross pathology and results of the histopathology indicate that RLOs induce a chronic granulomatous infection in the sea bass farmed in Turkey. The gross pathology observed in these fish bears similarities to that reported during rickettsial infections in other fish species [farmed salmonids in Chile (Fryer & Mauel, 1997), Atlantic salmon (Rodger and Drian, 1993; Lannan et al., 1991), Nile and Mozambique tilapia (Chen et al., 1994; Fryer and Mauel, 1997) and grouper (Chen et al., 2000)]. The most striking similarity is the multifocal grey to yellow nodules on the spleen and kidney. However, ring or crater-shaped, cream coloured lesions were not present on the liver as observed in chronically infected salmon and Nile tilapia (Turnbull, 1993; Rodger and Drian, 1993; Chen et al., 1994; Fryer and Mauel, 1997; Manuel and Miller, 2002).

![Figure 4. Immunohistochemistry on tissue sections from diseased fish using (a) rabbit anti-*Piscirickettsia salmonis* polyclonal antibody (liver); (b) monoclonal antibody against *Photobacterium damselae* subsp. *piscicida* (kidney). The presence of bacteria is indicated by dark staining in the tissue sections (mag x200).](image-url)
The histopathology also bore similarities to that observed in Nile tilapia (Chen et al., 1994), Atlantic salmon (Rodger and Drian, 1993; Turnbull, 1993) and blue-eyed plecostomus (Khoo et al., 1995), with the most striking similarity being multifocal necrosis of parenchyma cells, accompanied by chronic infiltration of mononuclear cells in the kidney, spleen, liver and in skeletal and heart muscle. The presence of fibrin thrombi, perivascular necrosis, chronic inflammatory cells with hypertrophy and RLOs laden cells as seen in the sea bass has also been reported in other fish species infected with RLOs. Most of the peripheral blood monocytes contained basophilic RLOs ranging from a few to many as described by other workers (Cvitanich et al., 1991; Noga, 2000; Lannan et al., 1999).

RLOs from European sea bass have been shown to share common antigens with the Piscirickettsia salmonis type-strain, LF-89, by indirect fluorescent antibody test (IFAT) and by IHC (Steiropoulos et al. 2002; McCarthy et al., 2005). In addition, DNA sequences of the 16S rDNA and 16S-23S internal transcribed spacer region (ITS) from the sea bass piscirickettsia-like organism are closely related to the salmonid pathogen P. salmonis (McCarthy et al., 2005). The same antiserum used by Steiropoulos et al. (2002) and McCarthy et al., (2005) was used here to identify RLOs in diseased fish.

The presence of mixed infections by Listonella (Vibrio) anguillarum or Photobacterium damselae subsp. piscicida and RLOs in fish from the three farms may have been an additional factor in accelerating the rate of mortalities in sea bass suffering from rickettsial disease.

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References


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