Infectious haemolytic anaemia causes jaundice outbreaks in seawater-cultured coho salmon, *Oncorhynchus kisutch* (Walbaum), in Chile

P A Smith[1], J Larenas[1], J Contreras[1], J Cassigoli[2], C Venegas[1], M E Rojas[1], A Guajardo[1], S Pérez[1] and S Díaz[1]

1 Unit of Pathology of Aquatic Animals, Department of Animal Pathology, Faculty of Veterinary Sciences, University of Chile, Santiago de Chile, Chile
2 Instituto Tecnológico del Salmón (INTESAL), Asociación de la Industria del Salmón (Salmón Chile), Puerto Montt, Chile

Abstract

In the last 9 years, epizootics of an icterus condition has affected coho salmon, *Oncorhynchus kisutch* (Walbaum), reared in seawater cages in southern regions of Chile. At necropsy, fish from field cases exhibited signs of jaundice accompanied by pale light-brown livers and dark spleens. Histopathological and haematological results indicated that these fish presented haemolytic anaemia. After microbiological examination no bacterial or viral agents could be identified as aetiological agents of this disease. In an infectivity trial, coho salmon, Atlantic salmon, *Salmo salar* L., and rainbow trout, *Oncorhynchus mykiss* (Walbaum), were inoculated intraperitoneally with a filtrate of an organ homogenate (0.45 μm) from a diseased coho salmon and held for 60 days in tanks supplied with fresh water. The disease was only reproduced in coho salmon in which mortalities, beginning at day 23 post-inoculation (p.i.), reached a cumulative value of 24% at day 27 p.i. This condition was transmitted to non-inoculated cohabiting coho salmon suggesting that it is a waterborne disease. Thus, this icteric condition is caused by an infectious form of haemolytic anaemia, probably of viral aetiology, and coho salmon are more susceptible than either Atlantic salmon or rainbow trout.

Correspondence *P A Smith, University of Chile, Faculty of Veterinary Sciences, Department of Animal Pathology, Unit of Pathology of Aquatic Animals, PO Box Casilla 2 Correo 15, La Granja, Santiago de Chile, Chile*
(e-mail: psmith@uchile.cl)

Introduction

Industrial farming of salmonid species in Chile began with the culture in sea cages of coho salmon, *Oncorhynchus kisutch* (Walbaum), in the late 1970s (Knut, Thorpe, Ridler, Monahan, Mahnken & Lindbergh 1993). Chile has been the world's largest producer of coho salmon since 1994 (Anonymous 2001) exporting 78 780 metric tons of this species in 2005 (Anonymous 2006). Historically, the most prevalent and economically important disease affecting coho salmon cultured in Chilean marine waters has been piscirickettsiosis (Fryer, Lannan, Garcés, Larenas & Smith 1990; Cvitanich, Gárate & Smith 1991; Smith, Rojas, Guajardo, Contreras, Morales & Larenas 2004). However, another disease has been progressively affecting coho salmon in sea cages since 1997 (Smith, Larenas, Contreras, Cassigoli, Venegas, Rojas, Guajardo, Troncoso & Macías 2002). This is an icterus condition which is colloquially referred to as 'guata amarilla' (yellow belly) and/ or 'síndrome ictérico del salmón coho' and occurs mainly in autumn in regions X and XI of southern Chile. Besides mortalities, which range between 1% and 30%, there are other associated economic losses such as delayed growth of the fish because farmers do not feed these animals as an empirical control measure against this disease. In this study,

the pathological condition was investigated using information from natural field outbreaks and from experimental infectivity trials.

Materials and methods

Field study

Sampling sites

Seven sea sites in southern Chile were sampled while presenting outbreaks of icterus in coho salmon. Six were located on Chiloé Island in Region X (sites ChI-1, ChI-2, ChI-3, ChI-4, ChI-5 and ChI-6) and the remaining site was in Aisén in Region XI (site A-1).

Fish sampling

From each site 15 moribund fish were killed, examined by necropsy and sampled for virology, bacteriology and histopathology. Individual samples of the kidney, liver and spleen were taken for bacterial and viral laboratory cultures. Kidney smears, fixed with pure methanol for 3 min, were also taken for Gram staining and fluorescent antibody tests (FAT) against *Renibacterium salmoninarum* (Laidler 1980) and *Piscirickettsia salmonis* (Lannan, Ewing & Fryer 1991). In addition, 26 fish (6, 5, 3 and 12 fish from sites ChI-1, ChI-2, ChI-3 and ChI-4, respectively) were bled (ethylenediaminetetraacetic acid 5 μg mL^{-1}) to obtain haematocrit and total blood haemoglobin concentration (Blaxhall & Daisley 1973), as well as blood smears which were fixed with pure methanol for 15 min before staining with Giemsa. Finally, from site ChI-6, sera from five icteric fish and from five healthy fish were obtained for measurement of total bilirubin (TB) by a photometric test (Diagnostic Systems, Holzheim, Germany) using 2,4-dichloroaniline (Rand & di Pasqua 1962).

Bacteriology

Fish tissues were cultured in trypticase soya agar (TSA), TSA + 1% NaCl and Cytophaga agar and incubated for 7 days at 17 °C.

Virology

For virological culture, organ pieces of the sampled fish were transported in Hanks' balanced salt solution supplemented with antibiotics and antimycotics and then seeded in CHSE-214 and SHK-1 cells, kindly provided by the late Prof. J.L. Fryer from Oregon State University (USA) and Dr B. Dannevig from the National Veterinary Institute (Norway), respectively. Cells were inoculated following the procedure of Kibenge, Gárate, Johnson, Arriagada, Kibenge & Wadowska (2001), except that the organ homogenates were diluted 1:100 instead of 1:50, and incubated at 17 °C for 21 days. When cytopathic effect (CPE) was observed cultures were tested by standard FAT to detect infectious pancreatic necrosis virus, infectious haematopoietic virus and viral haemorrhagic septicaemia virus (OIE 2000). In addition, spent supernatants from cell cultures showing CPE were tested using a reverse transcriptase-polymerase chain reaction (RT-PCR) assay to detect the infectious salmon anaemia virus (ISAV), as described by Mjaaland, Rimstad, Falk & Dannevig (1997). The RT-PCR tests were kindly carried out by Dr C. Cunningham at the Fisheries Research Services Marine Laboratory, Aberdeen, UK. In addition, samples of the liver, kidney, spleen and heart from selected coho salmon presenting typical clinical signs of this disease, both from the field ($n = 3$) and experimentally inoculated ($n = 4$), were preserved in RNA Later (Ambion, Austin, TX, USA) and also analysed by RT-PCR at the Marine Laboratory, to detect ISAV.

Histopathology

Samples of the kidney, spleen, liver, skeletal muscle, gills and heart were formalin fixed (8%, w/v) and processed to obtain 5-μm tissue sections which were stained with haematoxylin and eosin and with Prussian blue by standard procedures (Bruno & Poppe 1996).

Food analysis

Samples of the feed pellets used at the ChI-1 site were analysed to determine their *p*-anisidine and peroxide values (AOAC 1995).

Infectivity trials

Fish

Coho salmon ($n = 200$), Atlantic salmon, *Salmo salar* L., ($n = 200$) and rainbow trout, *Oncorhynchus mykiss* (Walbaum), ($n = 200$) of mean weight

45 (SD 19.6), 31.2 (SD 11.7) and 56 g (SD 15.1), respectively, were used. Fish were fed commercial pellets at 1% of their body weight daily and held in cylindrical tanks at a loading density of approximately 5 kg m^3. Tanks were supplied with a flow-through system of filtered fresh water (16 °C, SD 0.6).

Inoculum

A homogenate was prepared from a pool of organs (kidney, liver and spleen) obtained from a coho salmon presenting the typical clinical signs of the condition. This fish was collected from a sea-cage on Chiloé Island. The homogenate was diluted 1:100 (v/v) in minimal essential medium. The suspension was clarified by centrifugation at 41.4 *g* at 5 °C for 5 min, prefiltered at 5 μm and then filtered at 0.45 μm before use as an inoculum.

Experimental inoculation

Three groups of fish per species were used. One consisted of inoculated individuals, another of sham-inoculated fish that were held in cohabitation with the first group, and the remaining group was of sham-inoculated fish held in separate tanks. For each species four tanks were used. Twenty-five fish in one tank were injected intraperitoneally (i.p.) with 0.1 mL of the inoculum and held in cohabitation with 25 fish injected i.p. with 0.1 mL of phosphate buffered saline (PBS), pH 7.2. Fifty fish in another tank were injected i.p. with 0.1 mL PBS, pH 7.2. Fish in the remaining two tanks were replicates of the above. In the tanks where fish from two groups were held in cohabitation, individuals were marked by clipping in the left or right cartilage of the upper jaw to allow their group identification. Fish were observed for 60 days post-inoculation (p.i.). Necropsies were performed on dead fish and samples were taken from fresh mortalities to be analysed by bacteriology, virology and histopathology, as described above for the field study.

Results

Field study

Clinicopathology

In the seven sea sites inspected the disease only affected coho salmon although Atlantic salmon and rainbow trout were reared in the same sites. The earliest outbreak began 6 weeks after the smolts were transferred to sea. Weekly cumulative mortality ranged from 1% to 5% and the outbreaks lasted approximately 3–4 weeks. Water temperature while outbreaks occurred ranged from 12.1 to 14.2 °C (mean 13.18, SD 1.04). Sick fish showed a good body condition factor. Externally, most fish exhibited a yellowish colour in the skin of the abdominal region and bases of the paired fins, as well as in the periorbital tissues. In a few individuals, external pigmentation was golden/reddish. Internally, fish had pale gills, ascites, abundant visceral fat with yellow colour, a light-brown liver with a full gall bladder, dark spleen, pale kidney, hydropericardium and ventriculum covered by a fat layer and no food in the stomach or gut contents. In some cases the liver showed green patches with branched extensions. With histopathology, erythrocytes were scarce inside blood vessel lumens and there was an absence of haemorrhage in all the organs examined. Severe haemosiderosis accompanied by erythrophagocytosis was found in the kidney and spleen. Kidney haemosiderin was seen in macrophages of the haemopoietic tissue and in the epithelial cells of the proximal tubules (Fig. 1). All the livers exhibited moderate to severe diffuse steatosis and a few also had focal areas of acute necrosis. In some cases, livers also showed cholestasis. In some fish a degeneration of myocytes, resembling white muscle disease of mammals, was found in skeletal muscle and myocardium. The epicardium of all fish exhibited an extensive infiltration of adipose tissue and some also had foci of infiltrating mononuclear
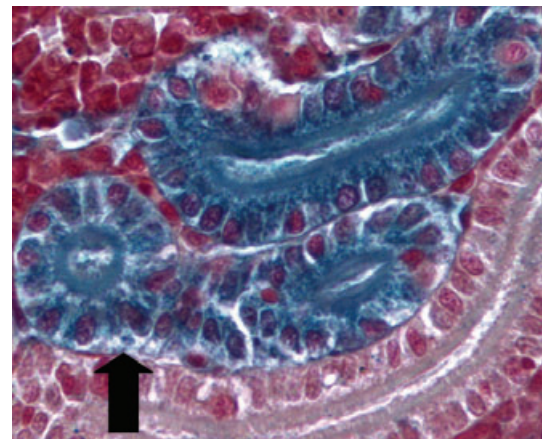
Figure 1 Haemosiderosis in the epithelial cells of the proximal tubules (black arrow) of a kidney of an icteric coho salmon collected from the field (Prussian blue, ×400).

cells. A few kidney samples presented vacuolar tumefaction and necrosis of tubular epithelial cells. The gills were normal.

Bacteriology

All cultures were negative. No bacteria were detected in kidney smears (Gram and FAT) except those of 11 fish from site ChI-4 which showed moderate levels of *P. salmonis*.

Virology

Most cultures were negative although a non-conspicuous CPE was observed in CHSE-214 and SHK-1 cells inoculated with samples from site ChI-4. This effect appeared on day 8 after cell inoculation and was characterized by the presence of foci of rounded cells that subsequently became detached. This culture was negative for all viruses tested by FAT and to ISAV by RT-PCR; no CPE was observed in three further blind passages in both cell lines.

Haematology

Haematocrit varied from 3% to 50% with a mean of 23% (SD 16.1). Total haemoglobin ranged from 1 to 10.5 mg dL^{-1} with a mean of 5.3 mg dL^{-1} (SD 3.7). No inclusion bodies were observed in blood smears stained with Giemsa, but these showed abundant immature erythrocytes. Sera of icteric fish were yellowish and had a TB mean of 0.12 mg dL^{-1} (SD 0.018), significantly higher ($P < 0.05$) than the values of healthy fish which were all below the lower detection limit of the test (< 0.07 mg dL^{-1}).

Food analysis

Peroxide and *p*-anisidine values were 4.8 and < 1 mEq kg^{-1}, respectively.

Experimental infectivity trial

The disease was reproduced in coho salmon but not in Atlantic salmon or rainbow trout. In the inoculated coho salmon mortalities began at day 23 p.i. reaching 24% cumulative mortality at day 27 p.i. (Fig. 2). Dead fish exhibited jaundice, ascites, dark spleen, hydropericardum and paleness of the gills and visceral organs with the livers
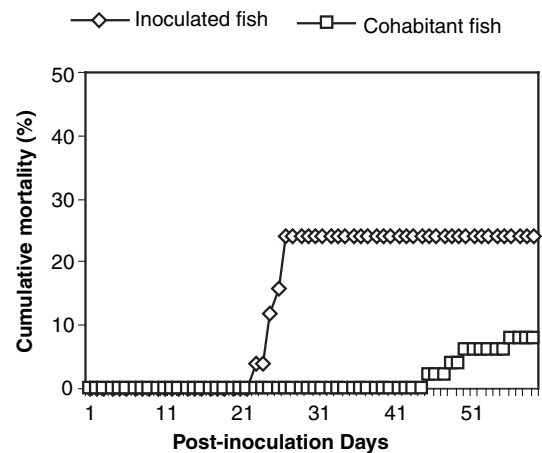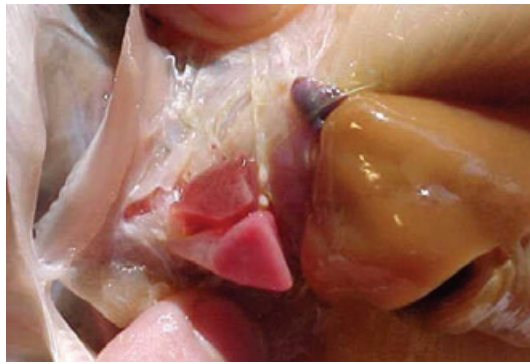

Figure 2 Cumulative mortality of coho salmon experimentally inoculated with an organ homogenate from an icteric fish.

Figure 3 Coho salmon showing jaundice after experimental inoculation with an organ homogenate from an icteric fish.

showing the same light-brown colour as the field cases (Figs 3 & 4). With histology all organs showed a low number of red blood cells inside the blood vessel lumens and an absence of haemorrhage. The spleen exhibited a massive haemosiderosis along with erythrophagocytosis. The disease occurred later in the coho salmon injected with PBS that were cohabitated with the inoculated fish. In this case mortalities began at day 46 p.i. and reached 8% at day 57 p.i. (Fig. 2). In the group of non-injected coho salmon which were not cohabitated with the inoculated group no deaths or clinical signs occurred throughout the experiment.

Bacteriological and virological cultures from diseased fish gave negative results. No bacteria were detected after of Gram staining and FAT (against *P. salmonis* and *R. salmoninarum*) of kidney smears

Figure 4 Coho salmon showing pale gills, heart and liver, after experimental inoculation with an organ homogenate from an icteric fish.

from sick fish. Samples of the kidney, spleen, liver and heart from four affected fish analysed by RT-PCR for ISAV were negative.

Discussion

Field epidemiological data and the experimental infectivity results indicate that coho salmon are susceptible to the disease and that Atlantic salmon and rainbow trout are resistant or have a lower susceptibility. The experimental reproduction of this icterus condition using filtered homogenates suggests a viral aetiology. No bacteria were found in experimentally infected fish and in the field samples *P. salmonis*, which was the only bacterium detected, was found in specimens from site ChI-4 only. This bacterium is endemic in salmonid populations in Chile and ChI-4 fish could have been affected by two different pathogens concurrently.

The haematological study showed reduced haematocrit and haemoglobin concentrations in the jaundiced fish with values as low as 3% and 1 mg dL^{-1}, respectively. The great number of immature erythrocytes in peripheral blood indicates an active response of the haemopoietic tissue to this anaemia. The significant increment of the TB values in the diseased fish confirmed that the yellowish pigmentation observed was caused by hyperbilirubinaemia. These haematological findings associated with the severe haemosiderosis and the lack of haemorrhage observed by histology, strongly suggest that these animals presented an acute haemolytic anaemia. Because the results of the study indicate that this disease is of an infectious aetiology, probably viral, we propose to name the condition as infectious haemolytic anaemia of salmon (IHAS).

The haemosiderosis found in the proximal tubular cells of the kidney (Fig. 1) suggests that, at least in part, the haemolysis may occur within the intravascular compartment of the fish. In mammals, the haemoglobin released after intravascular haemolysis is promptly bound by an α 2-globulin (haptoglobin) to produce a complex to prevent excretion into urine. When haptoglobin is depleted, free haemoglobin is filtered in the kidney glomeruli. The renal proximal tubular cells may reabsorb and catabolize much of this filtered haemoglobin. Iron released by the haemoglobin may accumulate, giving rise to haemosiderosis of the tubular epithelium. Concomitantly, the haem groups derived from the complexes are catabolized within the mononuclear phagocyte system, leading ultimately to jaundice (Cotran, Kumar & Collins 1999).

Large quantities of bilirubin found in the extracellular fluids of jaundiced individuals can commonly result from (1) increased destruction of red blood cells (haemolytic jaundice) and (2) damage to the liver cells or blockage of the bile ducts (obstructive jaundice) so that even the normal amounts of bilirubin cannot be excreted into the gastrointestinal tract (Guyton & Hall 1996). Although IHAS is a haemolytic-type jaundice, the liver lesions found in diseased fish suggest that an eventual hepatic failure may also be contributing to increase the bilirubin concentration in the blood. However, liver changes are more likely a consequence of the haemolytic anaemia and its associated hyperbilirubinaemia, rather than its cause.

A viral condition known as erythrocytic inclusion body syndrome (EIBS) has been associated with epizootics of anaemia in Pacific salmon (Leek 1987; Piacentini, Rohovec & Fryer 1989; Takahashi, Okamoto, Kumagai, Maita, Ikeda & Rohovec 1992). However, in the present study, no inclusion bodies were observed in erythrocytes from blood smears stained by Giemsa, which are usually detected in EIBS outbreaks (Takahashi *et al.* 1992). This suggests that the aetiological agent of IHAS is not the EIBS virus or that associated with viral erythrocytic necrosis, which also produces characteristic intra-erythrocytic inclusion bodies (Evelyn & Traxler 1978; Rohovec & Amandi 1981).

It should be noted that the ISAV was isolated from Chilean coho salmon showing jaundice (Kibenge *et al.* 2001) and serological evidence of its presence was also provided (Kibenge, Opazo, Rojas & Kibenge 2002). It may be that Chilean

coho salmon are suffering from clinically similar icteric syndromes that are caused by different aetiological agents, one of them being ISAV as reported by Kibenge *et al.* (2001).

The results of this study indicate that IHAS is produced by a filterable infectious agent but ISAV was not detected in samples either from the field or experimentally infected salmon. Although some similarities exist between the diseases, there are important differences in species susceptibility and in some of the pathological characteristics between ISA and IHAS. Atlantic salmon is highly susceptible to ISAV in marine culture in the northern Hemisphere (OIE 2003), but this species seems not to be affected in the field by IHAS. The higher susceptibility shown experimentally by coho salmon to IHAS relative to Atlantic salmon is different from that seen in ISAV trials where coho salmon proved to be more resistant to this virus than Atlantic salmon (Rolland & Winton 2003). On the other hand, the gross pathology of IHAS does not show the dark and enlarged liver or the petechiae in some visceral organs typically found in ISA (Thorud & Djupvik 1988; Evensen, Thorud & Olsen 1991; Hovland, Nylund, Watanabe & Endresen 1994). At a histological level, in fish affected by IHAS there is no haemorrhage and/or congestion in internal organs as frequently occurs in ISA (Evensen *et al.* 1991; Mullins, Groman & Wadowska 1998; Rimstad, Falk, Mikalsen & Teig 1999). In addition, the hepatocellular degeneration with an anastomising haemorrhage with intact tissue around the central veins, typically observed in later stages of ISA (Bruno & Poppe 1996), is not found in IHAS.

That diseased fish from the field had a good body condition factor together with liver steatosis and degeneration of muscular fibres suggested a hyperoxidation from rancid food ingestion was involved in the pathogenesis of this disease (Smith *et al.* 2002). However, food analysis, although limited to samples from only one affected site, showed no indication of rancidity and peroxide and anisidine values obtained were below 5 and 10 mEq kg^{-1}, respectively, which are considered as safe for salmonid diets (National Research Council 1993). Despite these results, further work is needed to determine if diet hyperoxidation also plays a role in the pathology of this disease in the field.

The results of the infectivity trials strongly suggest that IHAS is a waterborne disease as it was transmitted to cohabiting fish in fresh water. The fact that the disease has never been observed in fresh water in the field suggests that a marine reservoir of the aetiological agent may play a role in the epidemiology of IHAS. The putative viral agent of IHAS may not be well adapted to grow in CHSE-214 and/or SHK-1 cells and it may be necessary to develop new specific cell lines to facilitate its culture, as occurred in the case of the ISAV.

Acknowledgements

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