Piscirickettsia salmonis in farmed Atlantic salmon, *Salmo salar* in Scotland

Birrell, J., Mitchell, S. and Bruno, D.W.

Fisheries Research Services, Marine Laboratory, 375 Victoria Road, Aberdeen AB11 9DB Scotland UK

Abstract

The first report of a rickettsia-like bacterium in seawater farmed Atlantic salmon in Scotland was presented by Grant *et al*. (1996). During 2002, and reported here, the presence of *Piscirickettsia salmonis,* the causative agent of piscirickettsiosis in farmed Scottish salmon was confirmed by tissue pathology, ELISA and PCR. Gross lesions included an oatmealy-orange coloured liver with scattered creamy-coloured circular nodules. Histologically degeneration and necrosis of haematopoietic tissue and invasion by inflammatory cells occurred. Monthly losses were less than 1.51% per month.

Introduction

Piscirickettsia salmonis is recognised as the cause of a systemic disease of farmed marine rainbow trout, *Oncorhynchus mykiss* and salmon, *Salmo salar* after the first outbreak in southern Chile during 1989 (Bravo and Campos, 1989; Garcés *et al*., 1991). The causative agent is an obligate Gram negative intracellular bacterium and clinical and gross pathological changes associated with *P. salmonis* infections have been described (Cvitanich *et al*., 1991; Brocklebank *et al*., 1992 and Olsen *et al*., 1997). In Norway *P. salmonis* has been detected in farmed Atlantic salmon (Olsen *et al*., 1997). In addition, morphologically and serologically similar pathogenic rickettsia-like organisms (RLO) have also been reported in British Columbia in farmed pink salmon, *O. gorbuscha*, chinook salmon, *O. tshawytscha*, coho salmon, *O. kisutch* and Atlantic salmon (Brockelbank *et al*., 1992; Kent, 1992; Jones *et al*., 1998); and from Ireland and Scotland in Atlantic salmon (Rodger & Drinan, 1993; Grant *et al*., 1996). Since the latter case further sporadic outbreaks of RLO's have occurred in Scotland. In non-salmonids RLO's have been identified as the causative agent of outbreaks often with high mortality in pond reared tilapia, *Oreochromis* sp. in Taiwan and juvenile sea bass, *Dicentrarchus labrax* in France (Chern & Chao, 1994; Comps *et al*., 1996). Recently, Chen *et al*. (2000) found a *P. salmonis* -like bacterium associated with mortality of white seabass, *Atractoscion nobilis*. The review by Mauel *et al*. (2002) discusses this aspect in detail.

Currently the distribution of *P. salmonis* and related organisms are widespread and all cultured salmonid species are considered susceptible. However, the relationship between RLO's and *P. salmonis* requires further study.

Materials & Methods

Sampling

During a planned site visit to a fish farm in the Orkney Isles, Scotland in early October 2002, 150 Atlantic salmon were collected randomly from the sea cages. This visit was part of the European Union statutory programme

whereby all farms rearing animals for aquaculture are checked for certain infectious diseases. The fish were anaesthetised and portions of kidney removed from each fish to investigate infection by bacterial kidney disease (BKD), infectious pancreatic necrosis virus (IPNV), viral haemorrhagic septicaemia virus (VHSV) and infectious salmon anaemia virus (ISAV) in line with current legislation. Mortality data relating to the farm stock was noted and gross signs from two of the fish recorded. Tissues from two moribund fish were dissected (sample a), fixed in buffered formal saline and processed using standard protocols. Sections were stained with haematoxylin and eosin (H&E) and adjacent sections were Gram stained (Bruno & Poppe, 1996). During subsequent site visits in late October, November and March 2003 an additional 10, 5 and 2 moribund fish were sampled respectively (samples b, c and d).

Culture of Piscirickettsia salmonis

Portions of kidney were removed aseptically from the fish and stored at 4°C in a dry tube until processed. Tissues were homogenised at 1:20 in antibiotic-free balanced salt solution (BSS), without centrifugation. Tenfold dilutions were prepared at 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6} for inoculation onto cell culture. Monolayers of chinook salmon embryo cell line (CHSE-214, ECACC 91041114) were grown at 22°C in sealed flasks containing MEM supplemented with 2mM L-glutamine and 5% (v/v) foetal bovine serum. Isolates were propagated in CHSE-214 using antibiotic-free minimal essential medium (MEM) plus 5% bovine serum. The diluted tissue homogenates were inoculated directly (0.1ml/culture) into antibiotic-free culture medium overlaying the cells. The cell cultures were incubated at 15-18°C for 28 days and observed weekly for the appearance of cytopathic effect (CPE). If no CPE occurred (except in the positive control) cultures were incubated for an additional 14 days and then discarded as negative.

ELISA testing for Piscirickettsia salmonis

Using a commercial available ELISA test, salmon kidney tissue was diluted in extraction solution (1:2) homogenised, boiled for 10 minutes and centrifuged. The supernatant was added to duplicate wells of a microtitre plate coated with purified rabbit polyclonal antibody specific for *P. salmonis*. Plates were sealed to avoid evaporation of the reagents. Positive controls and negative controls were included and incubated for 1 hour at 37° ± 1°C before being washed to remove unbound material. A mix of the monoclonal and conjugate (1:1) was added and the wells resealed and incubated for 30 min at 37°C. After washing, an equal volume of tetramethylbenzidine and hydrogen peroxide was added and the plate incubated in the dark for 30 min at room temperature. Absorbance was measured with a MRX microplate reader fitted with a 450 nm filter.

Samples were considered positive when the absorbance was above the cut-off value, represented by the mean negative control value. Samples were considered negative when the absorbance was below the cut off value. If the absorbance of a sample was within the cut off value +/- 10% the sample was considered suspect.

DNA preparation

Piscirickettsia salmonis DNA was extracted from fish liver or from cell culture medium using DNAzol (Invitrogen, Paisley, UK) following the manufacturer's protocol. Briefly, 25-30 mg liver was homogenized in 1 ml DNAzol, cell debris was removed by centrifugation at 10000 x g for 10 min at 4°C. DNA was precipitated by the addition of 500µl absolute ethanol and incubation at room temperature for 5 minutes. The precipitate was pelleted at 4000 x g for 3 min at 4°C and washed twice with 1 ml 75% ethanol followed by centrifugation. The pellet was air dried then dissolved in 20µl 8mM NaOH. For tissue culture cells or supernatant, 100µl was added to 1 ml DNAzol.

Polymerase Chain Reaction (PCR)

The nested PCR method of Mauel *et al.* (1996) was used with minor modifications. This method utilises general bacterial 16s rDNA primers (Eub A, 5'-AAG GAG GTC ATC CAT CCA CA-3' and Eub B, 5'-AGA GTT TGA TCC TGG CTC AG-3') in the first amplification reaction. The second reaction employs *P. salmonis*-specific primers (Ps F, 5'-CTA GGA GAT GAG CCC GCG TTG-3' and Ps R, 5'-GCT ACA CCT GCG AAA CCA CTT-3'). 5µl DNA extract was added to 45µl of reaction mix consisting of : 1x PCR buffer (Bioline ammonia buffer), 2mM Mg Cl_2, 200µM each of dATP, dCTP, dTTP and dGTP, 2µM Eub A primer, 2µM Eub B and 2.5U Taq DNA polymerase (Biotaq, Bioline). The mixture was denatured at 94°C for 2 min and amplification was performed with 35 cycles of 94°C for 1 min, 50°C for 2 min and 72°C for 3 min. the tubes were then held at 4°C. The second amplification was performed by adding 3µl of the first PCR product to a reaction mixture similar to the first but containing 4µM each of primers Ps F and Ps R instead of EubA and EubB. Reaction conditions were 35 cycles of 94°C for 1 min, 61°C for 2 minutes and 72°C for 3 minutes. Ten µl of second PCR product was electrophoresed in 2% agarose containing ethidium bromide and photographed under UV transillumination. *P. salmonis* yields a band of 467 base pairs.

Results

During October 150 farmed Atlantic salmon were examined for statutory purposes (sample a). Two fish were lethargic, dark in colour and moribund, but no additional external lesions were recorded. Internally one fish showed an oatmealy-orange coloured liver with slight haemorrhage and scattered creamy-coloured circular areas throughout and a lighter coloured, 'grainy' kidney. Yellow faecal casts were noted in the gut lumen. The other fish showed reduced caecal fat body and yellow faecal casts. IPNV was confirmed in the farmed stock from this visit in October, and from the fish sampled in November, but not in March. No other disease agent was recorded.

Light microscopy

Histologically, a focal degeneration and necrosis of haematopoietic tissue and invasion by inflammatory cells was recorded from infected fish. In the liver several multifocal necrotic areas in the parenchyma with accompanying inflammatory response, dominance of polymorphonuclear granulocytes and scattered macrophages, perivascular inflammation and moderate necrosis occurred (Fig. 1). An intracellular location of Gram negative,

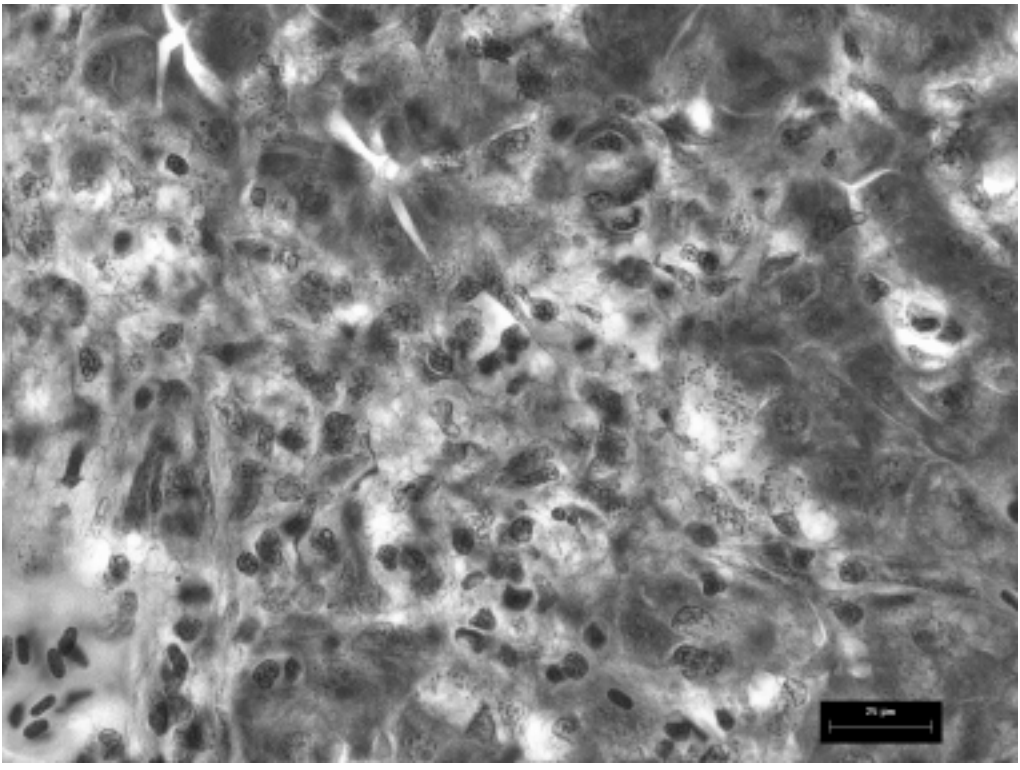

Fig. 1. Liver from Atlantic salmon infected with *Piscirickettsia salmonis*. Note multifocal necrotic areas in the parenchyma with intracellular location of bacteria. H&E staining. Bar scale =25µm.

short-rounded bacteria and hypertrophic macrophages were noted with a focal degeneration congestion of the ellipsoids. Epithelial hyperplasia in the gills was noted and in the pancreas mild thrombotic lesions.

ELISA and cell culture

One fish from150 (sample a) gave a positive ELISA result for *P. salmonis* from kidney tissue. Two out of ten fish (sample b) were positive by ELISA. *P. salmonis* was isolated on CHSE cell line and CPE was recognised easily. There was no evidence of *P. salmonis* using these methods in the November (sample c, 5 fish) or March sample (sample d, 2 fish).

Polymerase Chain Reaction (PCR)

Kidney samples reported as positive by ELISA were negative by PCR, however the cell supernatants from the culture samples were positive (2/10, sample b).

Mortality

Mortality at the Orkney site from all causes between October and March was 1.51%, 0.70%, 0.56%, 0.29%, 0.31% and 0.39% respectively.

Discussion

Piscirickettsiosis was first described in Chile in coho salmon in the late 1980's (Bravo & Campos, 1989) with subsequent mortality

above 90%. Later, Fryer *et al*. (1992) isolated the causative bacterium on CHSE and the organism was named *Piscirickettsia salmonis*. The report here identifies *P. salmonis* for the first time in Scottish farmed Atlantic salmon as confirmed by tissue pathology, ELISA and PCR. This follows from the first report of rickettsia-like organisms in Scottish salmon (Grant *et al*., 1996). The gross and light microscopical lesions described in the current study are similar to these previous reports of this disease (Cvitanich *et al*., 1991; Brocklebank *et al*., 1992; Olsen *et al*., 1997). The ELISA test was specific for *P. salmonis* and the CPE from the cell culture was distinct. A modification of the PCR method (Mauel *et al*., 1996) supported the above tests above and the isolation of *P. salmonis*.

The impact of the initial rickettsia-like outbreak in Scotland by Grant *et al*. (1996) reported obvious losses in the farmed fish although mortality data was not included. In this study mortality recorded at the farm site from all causes was less than 1.5% per month. Current data from this and other outbreaks, recorded as rickettsia-like, also suggest a relatively low impact in Scotland. Mortality from this disease in other countries has also not reached the importance and prevalence of the Chilean outbreaks. For example, 0.6% - 15% mortality has been reported in Canada and Norway (Brocklebank *et al*., 1992; Olesen *et al*., 1993). Evidence from this current outbreak suggests *P. salmonis* was a direct cause of chronic losses among the sea water-reared salmon. Although IPNV was diagnosed on this site by tissue culture and IPN is known to contribute to seawater mortality in farmed salmon (Smail *et al*., 1995), the histological lesions were not consistent with clinical IPN. Increasing losses from all causes were recorded in this study in October, but not in November or March of the following year. This is similar to the report by Grant *et al*. (1996) who noted mortality among the rickettsia-like infected stock in November.

Significant differences in relative virulence are reported for the Chilean type strain of *P. salmonis* (LF-89), Canadian (ATL-4-91) and a Norwegian strain (NOR-92) (House *et al*., 1999). Phylogenetic analysis demonstrated that strains from different geographic locations form a tight monophyletic cluster with 16S rDNA similarities ranged from 99.7 to 98.5% (Mauel *et al*., 1996). However, House *et al.* (1998) demonstrated that the Chilean strain was more pathogenic than strains from British Columbia and Norway, and there was evidence of at least two variants of the pathogen occurring in Chile and a factor that might account for mortality differences recorded in Scotland and elsewhere.

Acknowledgement

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