Characterisation of an emerging rickettsia-like organism in Tasmanian farmed Atlantic salmon *Salmo salar*

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ABSTRACT: A rickettsia-like organism (RLO) was observed in farmed Atlantic salmon *Salmo salar* located in south-east Tasmania, Australia. Several assays such as immunoperoxidase, immunoelectron microscopy, polymerase chain reaction and nucleic acid sequencing, as well as phylogenetic analysis of rDNA sequences, were performed on infected fish tissues. Immunohistochemistry results suggested the presence of related antigenic determinants between the Tasmanian RLO and the type strain LF-89 of *Piscirickettsia salmonis*. However, sequence alignment demonstrated that the Tasmanian RLO contains a 19 bp deletion at the 3'-end of the internal transcribed spacer region of the rDNA operon, indicating a genetic divergence from *P. salmonis* isolates, which are exotic to Australia.

KEY WORDS: Rickettsia-like organism · rDNA sequences · Differential antigenicity · *Piscirickettsia salmonis* · Atlantic salmon

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INTRODUCTION

Piscirickettsia salmonis, an obligate intracellular bacterial pathogen of salmonids, was identified as the aetiological agent of the disease known as piscirickettsiosis (Fryer et al. 1990, Fryer & Mauel 1997). *P. salmonis* was classified as a member of the Proteobacteria gamma subdivision based on 16S rRNA sequence (Fryer et al. 1992). Several salmonid species, including coho salmon *Oncorhynchus kisutch* and Atlantic salmon *Salmo salar*, are susceptible to infection. Outbreaks of the disease in farmed fish can cause substantial mortalities, leading to significant financial loss (Bravo & Campos 1989). To date, piscirickettsiosis has been identified in Chile (Bravo & Campos 1989), Ireland (Rodger & Drinan 1993), Norway (Olsen et al. 1997), Scotland (Grant et al. 1996, Birrell et al. 2003), and both the east (Jones et al. 1998) and west coasts of Canada (Evelyn 1992).

The recent detection of a rickettsia-like organism (RLO) through Giemsa staining and polymerase chain reaction (PCR) amplification from tissues taken from

farmed Atlantic salmon in Tasmania, Australia (Handlinger & Ellard pers. comm.) has identified a need to further characterise this RLO at the antigenic and molecular level in order to establish its relatedness to *Piscirickettsia salmonis* isolates exotic to Australia.

MATERIALS AND METHODS

***Piscirickettsia salmonis* isolates.** DNA from the Chilean (LF-89), Canadian (ATL-4-91) and Norwegian (NOR-92) reference strains of *P. salmonis* was obtained from cell culture supernatants as described previously (Corbeil et al. 2003) and used as positive controls for PCR amplification. The nucleotide sequences for the 16S rRNA gene or 16S–23S internal transcribed spacer region of *P. salmonis* LF-89 (GenBank accession numbers U36941 and U36943), NOR-92 (GenBank accession numbers U36942 and U36946), ATL-4-91 (GenBank accession numbers U36915 and U36945), SLGO-94 (Chile) (GenBank accession number U55015), EM-90 (Chile) (GenBank accession numbers

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U36940 and U36944), and C1-95 (Chile) (GenBank accession number U62103) were aligned and compared using the Clustal W algorithm (Thompson et al. 1994, BioManager from the Australian National Genomic Information Service, <http://www.angis.org.au>).

Tasmanian RLO isolates and DNA extraction. The RLO isolates used for genomic characterisation were harvested from 2 yr old moribund Atlantic salmon farmed in 2 different locations in the Huon Estuary D'Entrecasteaux Channel, South East Tasmania, over a 2 yr period (2001 to 2002). Liver, kidney and brain tissues from 1 fish in the first year and 2 fish from the following year were processed in order to compare the rRNA sequences of the RLO isolates. DNA was extracted from formalin-fixed and fresh salmon tissues (approximately 20 mg total tissue) by rinsing them in phosphate-buffered saline (PBS), followed by a digestion with Proteinase K and DNA elution using a QiaAmp DNA minikit (Qiagen) according to the manufacturer's instructions. DNA was eluted and resuspended in a final volume of 50 µl of sterile deionised water.

PCR amplification of the Tasmanian RLO rDNA genes. Partial regions of the 16S gene of the rDNA operon of the Tasmanian RLO were amplified following a method described by Mauel et al. (1996) using the PS2S and PS2AS primer set. The internal transcribed spacer (ITS) of the rDNA operon was amplified using methods described by Marshall et al. (1998), using the ITS1 and RTS4 primer set, and Casanova et al. (2001) using the universal primers L1 and G1 with the following modifications: the mixture was denatured at 94°C for 2 min and amplified by 35 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 40 s. The resulting amplicons were resolved by electrophoresis in 2% agarose gels in 1X TAE (Tris-acetate 0.04 M, EDTA 0.001 M) buffer. Gels were stained with ethidium bromide (0.5 µg ml⁻¹).

Sequence determination. The 16S rRNA partial gene and the ITS region sequences (GenBank accession numbers AY578984 and AY578985, respectively) were determined by direct sequencing of the PCR product. Sequencing was carried out using an ABI PRISM Ready Reaction Big Dye[®] Termination Cycle Sequencing Kit (Perkin-Elmer) and an ABI PRISM model 377XL DNA sequencer (Sequencing Analysis 3.4.1 Software, Version 2.6 [Perkin-Elmer]).

Phylogenetic analysis. Each Tasmanian RLO sequence was confirmed from 3 separate amplicons, each of which was obtained from different amplification reactions. The amplicons were sequenced in both forward and reverse directions. The Tasmanian RLO and *Piscirickettsia salmonis* sequences were aligned and phylogenetic trees were constructed from the

sequence data using the DNA Distance + Neighbor programs in PHYLIP Phylogeny Inference Package Version 3.2 (Felsenstein 1989, Biomanager, Australian National Genomic Information Service). For comparisons between the Tasmanian RLO and the *P. salmonis* isolates using the partial 16S rDNA gene and ITS region, 445 and 265 bp sequences were utilised, respectively, bootstrapped 100 times.

In vivo amplification of the Tasmanian RLO for cell culture. Three tissue samples originating from 1 Tasmanian farmed Atlantic salmon showing gross signs of RLO infection and testing positive for RLO by PCR were homogenised (10% w/v) in PBS without Ca²⁺ or Mg²⁺ ions (PBS-A; pH 7.4) using a mortar and pestle. Aliquots (200 µl) of tissue supernatants were injected into 30 g Atlantic salmon (obtained from Snob's Creek Fish Hatchery, Victoria, where the presence of RLO has never been observed). At 12 d post-inoculation, tissues from moribund fish as well as negative control fish were sampled and processed for cell culture isolation and laboratory examination.

Cell culture isolation. Liver and kidney tissues were aseptically removed from 11 experimental moribund fish (~30 g in weight) and were individually homogenised in 5 ml of antibiotic-free Eagle's Minimum Essential Medium (EMEM, Gibco) containing 10% (v/v) fetal bovine serum (FBS, Gibco). Aliquots (100 µl) of 10⁻¹ and 10⁻³ dilutions of the homogenates were used to inoculate duplicate 25 cm² monolayer cultures of 3 fish cell lines (chinook salmon embryo, CHSE-214; *epithelioma papulosum cyprini*, EPC; rainbow trout gonad, RTG-2). The monolayer cultures were incubated at 15°C for 30 d and monitored for cytopathic effects. Cell culture isolation was also attempted from 9 Tasmanian moribund farmed Atlantic salmon (2 yr of age) over a 4 mo period.

Immunoperoxidase assay. Formalin-fixed tissues were dehydrated and processed through to paraffin blocks using standard procedures. For each experiment, sections from negative control tissues from uninfected Atlantic salmon originating from Victoria, Australia, positive control tissues from coho salmon experimentally infected with the LF-89 isolate of *Piscirickettsia salmonis* in the USA and liver tissues from 6 moribund Tasmanian Atlantic salmon (2 yr old) were treated as follows. Following deparaffinisation, the sections were washed with PBS-A and then incubated for 1 h at 37°C with a polyclonal sheep anti-*P. salmonis* (LF-89) (Microtek) or normal sheep serum at a dilution of 1:100 in 1% (w/v) skim milk in PBS-A. After washing in PBS-A, the sections were incubated for 1 h at 37°C with a peroxidase-conjugated donkey anti-sheep IgG (Amersham) diluted 1:200 in 0.1% (w/v) skim milk in PBS-A. Following a further wash in PBS-A, the sections were incubated with freshly prepared substrate

solution: 2 mg 3 amino-9-ethyl carboxyzole (AEC) (Sigma), 200 µl dimethylformamide (VWR), 10 ml 0.05 M acetate buffer pH 5.0, 5 µl 30% (v/v) hydrogen peroxide (VWR) for 20 min at room temperature. The sections were rinsed in tap water, counterstained with Mayer's haematoxylin and mounted in GelTol (Immunon) for examination by light microscopy.

Preparation of ultrathin samples for electron microscopy. Sections of formalin fixed liver tissues from 1 Atlantic salmon (30 g in weight), originating from an experimental infection, embedded in paraffin and correlating to areas that were positive by immunoperoxidase assay, were cut from the blocks and processed as follows. Excised portions of the block were deparaffinised, washed in PBS-A prior to fixation in 2.5% (v/v) glutaraldehyde (90 min) in 0.1 M cacodylate buffer (pH 7.2), washed (3 × 20 min) in the same buffer, post-fixed in 1% (w/v) osmium tetroxide (1 h), rinsed (3 × 5 min) in water and dehydrated through a graded series of ethanol (20 min each). Specimens were then infiltrated and embedded in Spurr's epoxy resin or LR White Resin. Sections embedded in Spurr's resin were used for ultrastructural examination and those embedded in LR White Resin were used for immunoelectron microscopic examination.

Immunoelectron microscopy. Ultrathin sections were washed in PBS (3 min), incubated in PBS for a further 10 min and then placed in humid incubation chambers (Hyatt 1989, 1991) and incubated with the *Piscirickettsia salmonis* LF-89 isolate antiserum (diluted 1:200 in PBS) for 2 h at room temperature (22 to 24°C). Sections were then washed with PBS (6 × 3 min) and incubated with protein A-gold (10 nm particles) (diluted 1:100) at room temperature (22 to 24°C). Sections were subsequently washed with PBS, post-fixed with 2.5% cacodylate-buffered glutaraldehyde (5 min), rinsed in water (3 × 3 min) and stained with lead citrate and uranyl acetate. All specimens were examined at either 75 or 100 kV with a Hitachi H7000 scanning transmission electron microscope, calibrated with a 2160 lines mm⁻¹ grating replica.

RESULTS

Cell culture

In contrast to *Piscirickettsia salmonis* reference isolates LF-89, ATL-4-91 and NOR-92 obtained from other laboratories, inoculation of cell cultures with tissue homogenates from Tasmanian RLO infected moribund fish (either naturally infected farmed fish or experimentally infected fish) did not result in growth of the Tasmanian RLO. Inoculated cells remained healthy for the duration of the experiment.

Immunohistochemistry

The use of the sheep anti-*Piscirickettsia salmonis* antisera resulted in bright red staining for the homologous positive control tissues (Fig. 1) whereas the Tasmanian RLO infected tissue reacted only moderately (Fig. 2). RLO infected tissue stained with a non-immune sheep serum did not show any significant staining (Fig. 3).

Electron microscopy

Examination of ultrathin sections of RLO infected liver tissue showed coccoid pleomorphic organisms

Fig. 1. *Salmo salar*. Immunohistochemistry of *Piscirickettsia salmonis* (LF-89 isolate) infected coho salmon liver, using sheep anti-LF-89 *Piscirickettsia salmonis* polyclonal antibodies. Salmon tissue originates from an experimental infection. Magnification ×200

Fig. 2. *Salmo salar*. Immunohistochemistry of Tasmanian rickettsia-like organism infected Atlantic salmon liver, using sheep anti-LF-89 *Piscirickettsia salmonis* polyclonal antibodies. Salmon tissue originates from an experimental infection. Magnification ×400

Fig. 3. *Salmo salar*. Immunohistochemistry of Tasmanian rickettsia-like organism infected Atlantic salmon liver, using non-immune sheep polyclonal antibodies. Salmon tissue originates from an experimental infection. Magnification $\times 800$

possessing nucleoid regions containing electron-dense central areas, as well as an undulating outer cell membrane and second inner membrane (Fig. 4 & 5). The organisms varied in diameter from approximately 0.6 to 1 μm (Fig. 4). Examination of sections incubated with *Piscirickettsia salmonis* LF-89 antiserum and protein A-gold revealed the presence of weak but specific gold labelling. Labelling was restricted to the coccoid organisms. No labelling was observed outside of these areas (Fig. 5) or in uninfected tissue (Fig. 6).

Fig. 4. *Salmo salar*. Electron photomicrograph of Atlantic salmon liver infected with the Tasmanian rickettsia-like organism (arrows). Salmon tissue originates from an experimental infection

Fig. 5. *Salmo salar*. Immunoelectron photomicrograph of Atlantic salmon liver infected with the Tasmanian rickettsia-like organism. *Piscirickettsia salmonis* specific polyclonal antibodies gold beads (arrows). Bi-layer undulating membranes (arrowheads). Salmon tissue originates from an experimental infection

Fig. 6. *Salmo salar*. Electron photomicrograph of uninfected Atlantic salmon liver stained with the *Piscirickettsia salmonis* specific polyclonal antiserum and gold beads. Salmon tissue originates from an uninfected control group of an experimental infection trial

PCR amplification and sequencing analysis

The primer sets PS2S–PS2AS (16S region) and ITS-1–ITS-4 (ITS region) were used to amplify the Tasmanian RLO DNA. DNA from *Piscirickettsia salmonis* isolates LF-89, NOR-92, ATL-4-91 were used as positive controls. Primers specific for the 16S region produced amplicons of expected size (Mauel et al. 1996) for all samples tested (results not shown). The second PCR amplification using the ITS specific primers produced amplicons of the expected size (Marshall et al. 1998) for the *P. salmonis* isolates and an amplicon of smaller size for the Tasmanian RLO (result not shown). Sequencing revealed a deletion of 19 consecutive base pairs located at the 3'-end of the ITS sequence (Fig. 7). Two amplicons were obtained from the PCR amplification using the universal primers L1 and G1, performed on the LF-89 isolate of *P. salmonis* as well as on the Tasmanian RLO. Amplicon sizes were slightly different between the isolates (result not shown). Sequencing of the RLO PCR products showed that the larger amplicon was identical to the smaller one at the termini, but the sequence was interrupted by an insert of around 300 bp containing tRNA-Ala and tRNA-Ile as previously described by Casanova et al. (2001). The 2 tRNA sequences are identical between the RLO and the LF-89 and EM-90 isolates of *P. salmonis*, with the exception of a cytidine instead of a thymidine in position 61 of the RLO tRNA-Ala (results not shown).

Phylogenetic analysis

The phylogenetic analyses were performed using 445 bases from the 16S region and 265 bases from the ITS region including insertions and deletions. When the analysis was conducted using these 2 sets of sequences, both trees suggested that the EM-90 isolate of *Piscirickettsia salmonis* is most closely related to the Tasmanian RLO (Fig. 8) of the isolates included in this study.

DISCUSSION

Previous studies on this emerging disease of farmed Atlantic salmon in Tasmania concluded that a RLO was the aetiological agent (Handler & Ellard pers. comm.). In this study, electron microscopic observations of the Tasmanian RLO support the initial histological identification of a RLO in the fish tissues (Handler & Ellard pers. comm.). The weak immunohistochemical staining obtained with the Tasmanian RLO infected tissues suggests antigenic similarity with the LF-89 isolate of *Piscirickettsia salmonis*. The immunogold staining of the RLO supports the immunohistochemical staining result and also suggests that the *P. salmonis* antiserum (directed against the LF-89 strain) cross-reacts with the Tasmanian RLO, hence suggesting the presence of at least some shared anti-

	1	11	21	31	41	51	61	71	81	91	
consensus	TGATTTTATTGTTT	TAGTGAGA	ATGATATT	TGTTCTTT	TAACAATG	TGGTAAAA	AGTATA	AGTAAAGATTC	CTTGATTA	ATTAGGGTTATTTT	AGTTT G
LF_89											A.
C1_95											C.
SLGO_94											C.
ATL_4_91											A.
NOR_92											T...A.
EM_90											T...C.
RLO_Tasman											T.
	101	111	121	131	141	151	161	171	181	191	
consensus	GTTGAGATG	TATTTTAT	--GTCTTG	ATTGATGAT	ATTAGAAATA	ATTTTATG	TTTATTTA	ATTAAACG	AGTCTTG	GTAATTTT	TGAAACCGGTGTTGAGAT
LF_89											T.....
C1_95											T.....
SLGO_94											T.....
ATL_4_91											G.....AT...G.....G.....
NOR_92											G.....AT...G.....G.....
EM_90											A.....T.....A.A.G.G.....
RLO_Tasman											A.....A.....A..G.G.....
	201	211	221	231	241	251	261				
consensus	ATAATTTTGATT	GGTTT	TAGTTA	TAGATT	TATGAT	TATAAG	ACTTTT	TGGGGTTATAT			
LF_89											
C1_95											
SLGO_94											
ATL_4_91											
NOR_92											
EM_90											
RLO_Tasman											

Fig. 7. Comparison of the nucleotide sequences of the internal transcribed spacer (ITS) region amplified with primers ITS-1 and ITS-4, for 6 *Piscirickettsia salmonis* isolates and the Tasmanian rickettsia-like organism. Points indicate nucleotide identity with the consensus sequence. Dashes indicate nucleotide deletion

Fig. 8. Phylogenetic analysis of (a) 16S rRNA and (b) ITS rRNA sequences constructed using distance method (Jukes & Cantor). Bootstrap support from 100 replicates shown at nodes

genic determinants between the RLO and *P. salmonis*. Even though only weak labelling was observed on the RLO infected tissues, no gold particles were observed on host cells or structures or on uninfected fish tissues (Fig. 6), indicating that the labelling was specific. Weak antigenic cross reactivity of the *P. salmonis* polyclonal antiserum with the Tasmanian RLO is a possible explanation for the low level staining. In a similar study involving the isolation of a white sea bass (*Atractoscion nobilis*) RLO, Chen et al. (2000a) reported that a *P. salmonis* specific polyclonal antiserum also produced a weak cross-reaction with the white sea bass RLO. These data suggest the existence of RLOs that are not *P. salmonis*. Moreover, the inability to culture the Tasmanian RLO *in vitro* under the conditions employed in this study may suggest a further difference between this organism and *P. salmonis* isolates.

In order to characterise the Tasmanian RLO at the molecular level we targeted the 16S rRNA gene, which is now well-established as a standard genetic marker

for the identification of species, genera and families of bacteria (Woese 1987, Amann et al. 1995). In addition, it has also been demonstrated that the ITS region of the rRNA operon is useful to differentiate species of prokaryotes (Barry et al. 1991, Jensen et al. 1993). A previous study based on the analysis of the rRNA operon of 5 isolates of *Piscirickettsia salmonis* suggested that sequence data from 3 regions (16S-ITS-23S) provided similar phylogenetic information (Mauel et al. 1999). The 5 isolates under investigation were then found to be closely related to each other. However, the EM-90 Chilean isolate was described as unique, showing slightly lower percent sequence identity in its rRNA sequences. The authors concluded that this latter isolate had diverged genetically from the others. In our current study, a phylogenetic analysis performed on this partial gene sequence of the 16S rDNA indicated that the Tasmanian RLO is closely related to *P. salmonis*, and particularly to the Chilean isolate EM-90 (Fig. 8a). In addition, we chose to amplify and sequence the RTS-1/RTS-4 amplicon of the ITS region as it has been suggested as a promising region for monitoring genetic variation in *P. salmonis* (Marshall et al. 1998). This DNA region allowed us to further evaluate the genetic relatedness of the Tasmanian RLO with strains originating from other geographical regions. We found the ITS sequence of the Tasmanian RLO to be slightly less conserved than the 16S sequence when compared with the 5 exotic *P. salmonis* isolates (results not shown). The phylogenetic analysis performed on the ITS sequences also indicated that the Chilean isolate EM-90 found in Atlantic salmon is genetically the closest to the Tasmanian RLO (Fig. 8b). In order to further characterise the ITS region of the RLO, we performed a PCR amplification using the G1-L1 primer pair previously used by Casanova et al. (2001) with *P. salmonis*. Two tRNA sequences were also identified in the ITS region of the Tasmanian RLO. This genetic feature characteristic of *P. salmonis* isolates (Casanova et al. 2003) may be a basis to attribute the name of *P. salmonis*-like organism to the Tasmanian RLO.

The Tasmanian RLO shares morphological characteristics with *Piscirickettsia salmonis* (Fryer et al. 1990, Cvitanich et al. 1991, Rodger & Drinan 1993) as well as with other RLOs (Chen et al. 2000b). While the 16S rDNA sequence and phylogenetic analysis demonstrated that the Tasmanian RLO is related to exotic *P. salmonis* isolates (Fig. 8a), the Tasmanian RLO exhibits an ITS sequence divergence with the 5 *P. salmonis* isolates that were examined, principally due to a 19 bp deletion (Fig. 7), which suggests a genetic divergence from *P. salmonis*. Sequence analysis of the rDNA ITS region of different strains of *Escherichia coli* has shown that large variations are found at certain locations in

the ITS without apparent functional consequences (Anton et al. 1998) and this is probably also true among other members of Rickettsiales (Andersson et al. 1999). Certainly, compared with *P. salmonis*, the 19 bp deletion observed in the ITS region of the Tasmanian RLO has had no obvious effect on its pathogenic status in fish. Nonetheless, the relatively weak cross-reactivity of the Tasmanian RLO with *P. salmonis*-specific anti-serum suggests that although the Tasmanian RLO is related to *P. salmonis* found in other countries there may be genetic variation elsewhere in the genome that has affected the nature of at least some antigenic determinants.

Recently, a *Piscirickettsia*-like organism, causing disease in Hawaiian tilapia, was found to have many morphological and pathogenic characteristics in common with *P. salmonis*. However, this organism did not produce an amplicon when a *P. salmonis*-specific PCR was performed (Mauel et al. 2003). Furthermore, other RLOs have been reported worldwide in different fish species (Khoo et al. 1995, Chen et al. 2000a,b). These cases emphasise the diversity of RLOs existing in aquatic species as well as the current inadequacy in the classification of aquatic rickettsia. Gene sequences from RLOs found in other fish species around the world will undoubtedly help to shed some light on the existing diversity of RLOs and help establish an appropriate classification of these emerging aquatic pathogens as it is not yet clear how similar a RLO would need to be in order to be classified *P. salmonis*.

The origin of this Tasmanian RLO is unknown; however, recent development of Atlantic salmon farms along the coast of Tasmania could have provided a new ecological niche for the RLO that may be endemic in wild indigenous species.

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