FINAL REPORT

aquatic animal health subprogram

Aquatic Animal Health Subprogram: development of diagnostic procedures for the detection and identification of *Piscirickettsia salmonis*

Serge Corbeil and Mark St. J. Crane

April 2004

FRDC Project No. 2001/624

Australian Government
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NON TECHNICAL SUMMARY

2001/624 Aquatic Animal Health Subprogram: Development of Diagnostic Procedures for the Detection and Identification of *Piscirickettsia salmonis*

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OBJECTIVES:

Original Objectives

1. Import exotic strains of *P. salmonis* and specific reagents from the OIE reference laboratory and other laboratories. Explore opportunities for collaboration with these laboratories and draw on their international expertise.

2. Introduce and optimise a standard cell culture system for the isolation and growth of *P. salmonis*.

3. Introduce and optimise a PCR assay for the identification and distinction of *P. salmonis* strains (exotic) and the rickettsia-like organism (RLO) (enzootic) present in cell culture supernatant and/or in fish tissues.

4. Develop an immunoperoxidase assay using available specific monoclonal antibodies and/or polyclonal antisera for the identification of *P. salmonis* in fish tissues.

5. Compare the nucleic acid sequence of the emerging Tasmanian isolates of *P. salmonis* to sequences of known exotic strains.

6. Purify cell culture-derived *P. salmonis* and raise specific polyclonal antisera in rabbits.

7. Quantify the detection limit of *P. salmonis* by PCR.

Approved Revised Objectives

Objective 6
A molecular probe suitable for *in situ* hybridisation use on tissue sections will be developed, evaluated and made available to diagnostic laboratories.

NON TECHNICAL SUMMARY:

P. salmonis is a serious pathogen of salmonid fish species, which can cause significant losses in farmed salmonids. The development/establishment of an internationally recognised Standard Diagnostic Technique for the detection and identification of *P. salmonis* and related pathogens will permit State/Commonwealth agencies to establish accurate information on the presence or absence of these pathogens in fish populations. Thus the use of these procedures will play an important role in the management of any disease outbreak caused by *P. salmonis* or other RLOs.

Industry sectors that will benefit from these outcomes include salmonid aquaculture in Tasmania and other States. Identification of infected and uninfected stock will allow industry and State officers to implement a disease management plan based on accurate information. Isolation of infected and uninfected fish populations will be possible, thus enhancing our capability to control and/or eradicate the disease.

State diagnostic laboratories will benefit from being provided with sensitive and specific reagents and procedures that have been validated using both exotic and enzootic isolates. Based on accurate diagnoses, State officials responsible for conducting activities of the Local Disease Control Centre (LDCC) will be able to make informed decisions during implementation of disease management procedures.

Piscirickettsiosis, caused by *P. salmonis*, is listed by the OIE and, as a Member Country, Australia is obliged to report on its presence or absence in Australia. Such reporting can only be achieved if there are sensitive and specific reagents and procedures in place that are recognised by international agencies. Using internationally accepted procedures Australia can be confident of providing accurate information to the OIE, other international agencies and to our international trading partners.

Sequence data comparing the Tasmanian isolate with other exotic isolates will assist in determining the relationship between the Tasmanian isolate and these exotic and pathogenic strains. Such data will be useful in identifying any new variants which may occur in the future as well as identifying any incursion of potentially highly pathogenic strains. Again, this type of information will allow appropriate management procedures to be put in place, as required.

Procedures developed during the course of this project have already been used to assist DPIWE Tasmania, and industry, in the management of the occurrence of an RLO in farmed Atlantic salmon. Diagnostic procedures have been transferred to the Fish Health Unit, DPIWE, Tasmania.

KEYWORDS: Piscirickettsiosis; Atlantic salmon; diagnostic procedures

ACKNOWLEDGEMENTS

The authors acknowledge the assistance of the State fish veterinarians from DPIWE, Tasmania. Discussions with State officers and their provision to AFDL of samples from fish with clinical signs of piscirickettsiosis greatly facilitated the research.

BACKGROUND

Piscirickettsia salmonis is the aetiological agent of the salmonid disease piscirickettsiosis. It is an intracellular Gram-negative bacterium that replicates within membrane-bound cytoplasmic vacuoles in the cells of infected fish. It is related to the genera *Coxiella* and *Francisella* and is grouped with the gamma subdivision of the Proteobacteria (Fryer and Mauel, 1997). Thus far, the disease piscirickettsiosis has been described in Chile (Bravo and Campos, 1989), Ireland (Rodger and Drinan, 1993), Norway (Olsen et al., 1997), and both East (Jones *et al*., 1998) and West Canada (Evelyn, 1992). Onset of disease commonly occurs following transfer of fish from freshwater to seawater holding facilities. Reported losses range from 30 to 90% mortality (Bravo and Campos, 1989; Fryer *et al*., 1990; Cvitanich *et al*., 1991) in the Chilean mariculture industry. The disease has also been observed in freshwater facilities (Bravo, 1994; Gaggero *et al*., 1995).

Coho salmon (*Oncorhynchus kisutch*) are believed to be the more susceptible salmonid species, however, *P. salmonis* has also been detected in chinook salmon (*O. tshawytscha*), sakura salmon (*O. masou*), rainbow trout (*O. mykiss*), pink salmon (*O. gorbusha*), and Atlantic salmon (*Salmo salar*). Non-salmonid species can also be infected with *P. salmonis*, as the bacterium was recently isolated from grouper (*Epinephelus melanostigma*) cultured in Taiwan (Chen *et al*., 2000a) and from cultured white seabass (*Atractoscion nobolis*) in USA (Chen *et al*., 2000b). Affected fish may appear dark and lethargic, hanging at the net sides. The first physical evidence of disease may be the appearance of small white lesions or shallow haemorrhagic ulcers on the skin. The major pathological changes are gill pallor, peritonitis, ascites, enlarged spleen, swollen grey kidney and liver with pale necrotic lesions (Cvitanich *et al*., 1991; Fryer et al., 1990; Olsen *et al*., 1997; Rodger and Drinan, 1993).

P. salmonis does not replicate on bacteriological media, but instead must be grown in cell culture, and therefore escapes detection by routine techniques used for bacterial isolation. Additionally, *in vitro P. salmonis* is sensitive to many antibiotics used in routine virus isolations (Lannan and Fryer, 1991) and will not grow even if inoculated onto suitable host cells if such compounds are included in the culture medium.

The 16S rDNA, 23S rDNA and internal transcribed spacer (ITS) regions have been sequenced in order to distinguish *P. salmonis* isolates at a molecular level. Based on the analysis of single isolates, some minor genetic variations were detected between geographic regions of the world (Mauel *et al*., 1999). Virulence differences among three isolates (LF-89, Chile; ATL-4-91, British Columbia, Canada; NOR-92, Norway) were demonstrated under controlled laboratory conditions, with the Chilean isolate, LF-89 being the most virulent in coho salmon (House *et al*., 1999). Although the isolates are genetically closely related, there are clearly factors that differ among them that affect how they interact with the host.

Laboratory diagnosis of *P. salmonis* infection is based on detection of the bacterium in Giemsa stained tissue sections or impressions and/or its isolation in cell culture (Fryer *et al*. 1990; Cvitanich *et al*., 1991; Lannan and Fryer, 1991). Identification is confirmed by indirect fluorescent antibody test (IFAT) (Lannan *et*

al., 1991) or polymerase chain reaction (PCR) (Mauel *et al*., 1996, OIE 2003). An immunohistochemical diagnostic test developed by Alday-Sanz *et al*. (1994) can be used to detect *P. salmonis* in formalin fixed tissues using monoclonal antibodies now commercially available.

The recent first detection of Rickettsia-like organisms (RLOs) in Giemsa stained tissue of farmed Atlantic salmon in Tasmania (unpublished) has highlighted the need for development of specific and reliable methods for identification of *P. salmonis*. Currently, neither diagnostic reagents nor experimental procedures are available in Australia that would allow isolation and specific identification of *P. salmonis*.

It is anticipated that significant outcomes of the proposed project will include development of reagents and procedures for the specific and sensitive detection, isolation and identification of *P. salmonis* from farmed salmonids. Furthermore, the use of molecular tools such as PCR and gene sequencing will allow for the comparison of *P. salmonis* isolates emerging in Australia with exotic strains of known virulence. This information will form the basis for the development of a Standard Diagnostic Technique for this aquatic animal pathogen, as part of AQUAPLAN.

NEED

Procedures and reagents for the diagnosis of *P. salmonis* are not currently available in Australia and are required to ascertain the presence or absence of infection in domestic farmed salmonids. The inability to determine quickly and reliably whether *P. salmonis* is present in fish and the inability to anticipate the degree of pathogenicity (high virulence vs. low virulence) of the recently isolated Tasmanian strain is a significant deficiency in our capability to manage the presence of this pathogen that could cause serious loss to the aquaculture industry.

It is essential to develop reagents and procedures for the isolation and identification of *P. salmonis* (cell culture, polyclonal antisera, and immunohistochemistry) and related organisms (RLOs), as well as modern molecular biology techniques (PCR, gene sequencing) that have the ability to quickly differentiate strains of known pathogenicity (House *et al*. 1999; Mauel *et al.* 1996, 1999). Without development of these reagents and procedures, it will be difficult to monitor and control the emergence and spread of *P. salmonis*.

OBJECTIVES

Original Objectives

1. Import exotic strains of *P. salmonis* and specific reagents from the OIE reference laboratory and other laboratories. Explore opportunities for collaboration with these laboratories and draw on their international expertise.

2. Introduce and optimise a standard cell culture system for the isolation and growth of *P. salmonis*.

3. Introduce and optimise a PCR assay for the identification and distinction of *P. salmonis* strains (exotic) and the RLO (enzootic) present in cell culture supernatant and/or in fish tissues.

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Approved Revised Objectives

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A molecular probe suitable for *in situ* hybridisation use on tissue sections will be developed, evaluated and made available to diagnostic laboratories.

METHODS

Importation of Reference Materials

Exotic strains of *Piscirickettsia salmonis*

Overseas laboratories were contacted to source reference strains of *Piscirickettsia salmonis*, import permit obtained (Import Permit Number 200010594), and actively growing cultures (in freshly infected CHSE-214 cell cultures) of the Chilean (LF-89), Canadian (ATL4-91) and Norwegian (NOR-92) isolates of *P. salmonis* were kindly supplied by the OIE reference laboratory (Dr J. L. Fryer, Oregon State University, USA). All exotic strains were held in the microbiologically secure laboratory at the Australian Animal Health Laboratory (AAHL) in Geelong and all subsequent investigations were carried out in the biosecure facility.

Tasmanian Rickettsia-like Organism (RLO)

Fresh and fixed tissues from RLO-infected Atlantic salmon farmed in Tasmania were obtained with the cooperation of staff from the Department of Primary Industries, Water & Environment, Tasmania. All diagnostic tests established during the course of this project were applied to the exotic strains of *P. salmonis* as well as the Tasmanian RLO.

Immunodiagnostic Reagents

During the course of the project it was noted that a commercial polyclonal antiserum was available for use in the diagnosis of Piscirickettsiosis:

Anti-*P. salmonis* (LF-89 isolate) sheep polyclonal antiserum (Microtek International; Cat # SPS01)

This antiserum was imported and its diagnostic application (immunohistochemistry and immunocytochemistry) was evaluated under normal conditions at AAHL.

Innocuity and Purity Testing

The identity and purity of the imported isolates were established according to procedures outlined in the AAHL Microsecurity Manual prior to any development work being initiated.

Bacterial DNA Isolation

DNA from *P. salmonis*-infected cell supernatants and from uninfected CHSE-214 cells was extracted using a QiaAmp DNA minikit (Qiagen) and Clontech spin column (Clontech) according to the manufacturer's instructions. DNA bound to minicolumns was eluted then resuspended in a final volume of 100μL of sterile deionised water. Extracted DNA was used for PCR analysis using primers specific for a number of different micro-organisms.

PCR Analysis

PCR analysis, using *P. salmonis*-specific primers (Marshall *et al*. 1998), was undertaken on each of the reference strains. A nested PCR, using general bacterial 16S rDNA primers in the first amplification reaction and *P. salmonis* specific primers in the second reaction (OIE, 2003), was performed on the extracted DNA.

In order to detect any significant viral pathogens of fish, RNA was also extracted from the *P. salmonis* isolates using QIAamp viral RNA mini kit and then RT-PCR assays, specific for IPNV, IHNV, and VHSV, were undertaken on the *P. salmonis* extracted samples.

Bacteriological Culture

Using standard bacteriological procedures, samples of the imported materials were plated on blood agar and incubated at 20^{O}C for 4 days in an attempt to isolate any contaminating bacterial pathogens of fish.

Experimental Infections

In order to generate sufficient material to support research and development, experimental infection of Atlantic salmon was undertaken.

Fresh fish tissues (kept on ice) originating from Tasmania were tested by PCR for the presence of RLO. Positive samples were used as a source of infectious material. Approximately 1g of liver and kidney tissues were mashed in 10mL EMEM (containing 10% FCS). The suspension was centrifuged at 100 x g for 1 min at 4^{O}C. The supernatant was kept as source of RLO suspension and stored at 4^{O}C until use.

Fifty Atlantic salmon (approximately 20g in weight), sourced from Snob's Creek Fish Hatchery, Alexandra, Victoria, were transferred to AAHL, treated for external parasites and acclimatised to conditions at AAHL.

For inoculation fish were anaesthetised using 10mg/mL ethyl-4-aminobenzoate. Thirty-five fish were injected with 200µL of the suspension containing the Tasmanian RLO. Twelve control fish received 200µL of medium alone.

Control and experimental groups were maintained in 150L freshwater at a temperature of 18^{O}C and were fed everyday. To maintain water quality, 1/3 of the water volume in each tank was replaced with new water each day.

Fish were monitored for clinical signs. Moribund fish were sampled immediately for laboratory investigation (cell culture and PCR).

Cell Culture System for the Isolation and Growth of *P. salmonis*

Fish Cell Line Susceptibility

Standard fish cell lines, CHSE-214, FHM, EPC, RTG-2, BF-2 (Crane and Williams, 2000), were tested for their susceptibility to infection by the three reference strains of *P. salmonis*. Cultures of each of the cell lines were established in 25cm^2 flasks containing minimum essential medium (Gibco) supplemented with 2mM L-glutamine (Thermo Trace), 10% (v/v) foetal bovine serum (Gibco), and buffered to pH 7.5 with sodium bicarbonate (Gibco). These cultures were inoculated with 100µL tissue culture supernatant from previously *P. salmonis*-infected cell cultures, imported into AFDL. All cultures were incubated at 15^{O}C and monitored, by light microscopy, for the appearance of *P. salmonis*-specific CPE.

Quantification of *P. salmonis* Growth in Cell Culture

P. salmonis was harvested at 15 to 20 days post-inoculation when the cell culture monolayers showed complete cytopathic effect (CPE). Two independent cultures of the LF-89 isolate were grown and endpoint dilution assays were performed for each culture (in triplicate per culture). The tissue culture infectious dose 50% ($TCID_{50}$) was used to determine the infectivity titre of the inocula of the LF-89 isolate. The 96-well plate (Nunc) cultures in the endpoint dilution assay were incubated at 15^{o}C for 28 days, and then visually inspected for the presence of CPE. Dilution endpoints were determined by the method of Reed & Muench (1938).

In vitro Culture of the Tasmanian RLO

Liver and kidney tissues were aseptically removed from experimental moribund fish and were homogenised in 5mL of antibiotic-free Eagle's Minimum Essential Medium (EMEM, Gibco) containing 10% (v/v) fetal bovine serum (FBS, Gibco). Aliquots (100µL) of 10^{-1} and 10^{-3} dilutions of the homogenates were used to inoculate 25-cm^2 monolayer cultures of three fish cell lines (chinook salmon embryo, CHSE-214; *epithelioma papulosum cyprini*, EPC; rainbow trout gonad, RTG-2). The monolayer cultures were incubated at 15^{o}C for 30 days and monitored for cytopathic effects.

Submissions of fresh tissues from RLO-infected Tasmanian Atlantic salmon were processed for RLO isolation in cell culture.

Use of Antibiotics

The OIE recommends that antibiotics should not be used in cell culture medium. However, due to the nature of the samples submitted from Tasmania, studies on antibiotic sensitivity have been undertaken in an attempt to identify antibiotics which could be used in the cell culture system employed to isolate RLOs. A range of antibiotics and antimycotics were used to supplement the cell culture medium and their affect on *P. salmonis* growth and development was assessed by observation using light microscopy, as follows.

CHSE-214 cells were established in 24-well culture plates (2.4 x 10^5 cells per well). Following overnight incubation at 20^{O}C, the antibiotic-free medium was discarded and 100µL of *P. salmonis* (NOR-92 isolate) suspension ($10^{4.75}$ $TCID_{50}$/mL) were added to each well (uninfected control wells excepted). Plates were centrifuged at 100 x g for 3 min. and then incubated at 15^{O}C for one hour. Nine hundred µL of complete medium containing one of the antibiotics (4 wells per antibiotic) to be tested were added to each well. Uninfected cells were also used to evaluate the any toxic effect of antibiotics on the cells. Cultures were incubated for a period of 17 days and examined every 3 days for the appearance of *P. salmonis*-specific CPE. Experiments were carried out in duplicate. Qualitative assessment was made to determine the susceptibility of antibiotics on *P. salmonis* growth.

Purification of Cell Culture-derived *P. salmonis* for Polyclonal Antiserum Production

The availability of a commercial diagnostic antiserum for *P. salmonis* negated the necessity to raise our own diagnostic antiserum. Thus the commercial antiserum was evaluated for its diagnostic capability. In addition, the feasibility of raising an antiserum specific for the Tasmanian RLO was investigated. Since the Tasmanian RLO cannot be grown in the current cell culture system the feasibility of using subtractive hybridisation to identify gene products to use as antigens for antiserum production was investigated.

Subtractive Hybridisation

Description of method

Subtractive hybridisation is a powerful technique that can be used to identify nucleic acid sequences that are present in one sample but absent in another (Duguid *et al*., 1990; Wieland *et al*., 1990). For example, the technique could be used to identify the presence of nucleic acid sequences of a pathogen present in tissue of an infected host without any prior knowledge of the pathogen's genome.

The basic theory behind subtraction is as follows:
The genomic DNA sample that contains the sequences of interest is called "tester" (e.g. the pathogen in the example above), and the reference sample is called "driver" (e.g. the host in the example above). Tester and driver DNAs are hybridised, and the hybrid sequences are then removed. Consequently, the remaining unhybridised DNAs represent tester-specific sequences.

Commercial kits are available for the application of this technology to various systems. At AAHL, expertise with the use of the Clontech PCR-Select™ Bacterial Genome Subtractive Kit (Clontech Laboratories, Palo Alto, California, USA) is available and this was used in an attempt to identify gene sequences of potential value in producing diagnostic antiserum.

PCR-Select subtraction applied to the Tasmanian RLO

Using the commercial kit (Clontech PCR-Select™ Bacterial Genome Subtractive Kit User Manual, PT 3170-1. Clontech Laboratories, Palo Alto, California, USA), genomic DNA was isolated from two fish tissue samples, one RLO-infected and the other uninfected. These tester and driver DNAs, respectively, were digested with the appropriate four-base-cutting restriction enzyme. The tester DNA was then subdivided into two portions, each of which was ligated with a different adaptor. The ends of the adaptors were unphosphorylated, so only one strand of each adaptor attached to the 5' ends of the DNA. The two adaptors have stretches of identical sequence, which allows annealing of the same PCR primer to both ends once the recessed ends have been filled in.

Two hybridizations were then performed. In the first, an excess of driver was added to each adaptor-ligated tester sample. The samples were then heat denatured and allowed to anneal. After this process single strand molecules presenting both types of adaptors were enriched.

During the second hybridization, the two primary hybridization samples were mixed together without denaturing. This ensured that only the remaining subtracted single strand tester DNAs could reassociate and form new types hybrids (positive strand with adaptor 1 and negative strand with adaptor 2R). These new hybrids were double stranded tester molecules with different ends. After DNA polymerase has filled in the ends, the double stranded tester molecules have different primer annealing sites on their 5' and 3' ends.

The entire population of molecules was then subjected to PCR to amplify the tester-specific sequences. The subtracted DNAs can be inserted into a cloning vector. Then, tester-specific DNA fragments can be identified by sequence and hybridization analysis.

Identification of *P. salmonis* and other RLOs by Immunoperoxidase Assays

Detection of *P. salmonis* in Cell Cultures by Immunocytochemistry

Commercial monoclonal antibodies specific for *P. salmonis* are not available. Therefore the objective of establishing an immunoperoxidase assay for detection of *P. salmonis* in cell cultures was achieved using a commercial polyclonal antiserum. Monolayers of CHSE-214 cell line were inoculated with *P. salmonis* and fixed 4 days later using cold acetone. An immunocytochemical reaction (immunoperoxidase assay) was established and optimised for the detection and identification of *P. salmonis* in cell cultures (see Appendix 3 for experimental details).

Detection of *P. salmonis* in Fixed Tissues by Immunohistochemistry

Paraffin blocks of fixed infected fish tissues, received from the USA, were used to validate the immunoperoxidase assay in fish tissues. Tissue sections cut from this block were processed for a standard immunoperoxidase test using the commercial

polyclonal antiserum as the primary antibody (see Appendix 3 for experimental details).

In order to determine whether the immunoperoxidase assay could identify the Tasmanian RLO in fish tissues, Atlantic salmon were inoculated with the Tasmanian RLO strain and sacrificed at various times post-inoculation for laboratory investigation. Tissues from internal organs were obtained and fixed in phosphate-buffered formalin.

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 *P. salmonis* in the USA (kindly donated by Dr M. House, Northwest Indian Fisheries Commission, Olympia, WA, USA and by Dr James Winton, USGS, Western Fisheries Research Center, USA), and tissues from moribund Tasmanian Atlantic salmon were treated as follows. Following deparaffinisation, the sections were washed with PBS-A and then incubated for 1 hour at 37^{o}C with a polyclonal sheep anti-*P. salmonis* (LF-89) (Microtek) or normal sheep serum at a dilution of 1:100 in 1% skim milk in PBS-A. After washing in PBS-A, the sections were incubated for 1 hour at 37^{o}C with a peroxidase-conjugated donkey anti-sheep IgG (Amersham) diluted 1:200 in 0.1% skim milk in PBS-A. Following a further wash in PBS-A, the sections were incubated with freshly prepared substrate solution (Table 1) 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.

Table 1. Immunoperoxidase Substrate Solution

Component	**Source**	**Weight/volume/concentration**
3 amino-9-ethyl carboxyzole; AEC	Sigma	2mg
Dimethylformamide	BDH	200µL
0.05 M acetate buffer pH 5.0		10mL
30% (v/v) hydrogen peroxide	BDH	5µL

PCR Assay for the Identification and Differentiation of *P. salmonis* Strains

Nested PCR

Samples for PCR analysis were prepared from cell cultures infected with imported *P. salmonis* strains, as well as fish tissues obtained from Atlantic salmon inoculated with the Tasmanian RLO strain. The nested PCR method recommended by the O.I.E. (O.I.E., 2003; Mauel *et al*., 1996) was used with some minor modifications.

DNA was released from formalin-fixed and fresh salmon tissues (liver, kidney, brain) by rinsing them in phosphate buffered saline (PBS) and extracted by using a QiaAmp DNA minikit (Qiagen) according to the manufacturer's instructions. DNA was eluted and resuspended in a final volume of 50µL of sterile deionised water.

Hot Start Master Mix (Qiagen) was used for both primary and nested PCRs. The generic primers Eub-A and Eub-B were used at a final concentration of 1µM in the primary reaction and the specific *P. salmonis* primers PS2S and PS2AS were used at a final concentration of 1µM in the nested reaction. Aliquots (2µL) of DNA sample were added to 23µL of reaction mixture for the primary amplification, and 2µL of the first amplified product were added to 23µL of reaction mixture for the secondary amplification. Thermo cycling conditions for the primary PCR were: 95^{O}C for 15 min (1 cycle), followed by 94^{O}C for 30 sec, 50^{O}C for 40 sec and 72^{O}C for 40 sec (35 cycles), followed by 72^{o}C for 5 min (1 cycle). Nested PCR cycling condition were: 95^{o}C for 15 min (1 cycle), 94^{O}C for 30 sec, 61^{O}C for 40 sec and 72^{O}C for 40 sec (35 cycles), followed by 72^{O}C for 5 min (1 cycle). PCR products were analysed by electrophoresis on a 2% agarose gel containing 0.5µg/mL ethidium bromide.

Single-step PCR

A single step PCR (Marshall *et al*., 1998) was also optimised in order to amplify the internal transcribed spacer (ITS) of the rDNA operon of the Tasmanian. Hot Start Master Mix (Qiagen) was used for the amplification. The specific *P. salmonis* primers ITS-1 and ITS-4 were used at a final concentration of 1µM. Aliquots (2µL) of DNA sample were added to 23µL of reaction mixture for the amplification. Thermo cycling conditions for the PCR were: 95^{O}C for 15 min (1 cycle), followed by 94^{O}C for 30 sec, 50^{O}C for 30 sec and 72^{O}C for 30 sec (35 cycles), followed by 72^{O}C for 5 min (1 cycle).

PCR products were analysed by electrophoresis on a 2% (w/v) agarose gel containing 0.5µg/mL ethidium bromide.

Comparison of the Nucleic Acid Sequence of the Emerging Tasmanian RLO Isolates to Sequences of Known Exotic Strains of *P. salmonis*

Sequence Determination

Partial 16S rRNA gene sequence and partial ITS region sequence 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 *P. salmonis* sequences were aligned and phylogenetic trees were constructed from the sequence data using the DNA Distance + Neighbour programs in PHYLIP Phylogeny Inference Package Version 3.2 (Felsenstein, 1989 and Biomanager). For comparisons between the Tasmanian RLO and the *P. salmonis* isolates using the partial 16S rDNA gene and the partial ITS region, 445 and 265 bases were utilized respectively, bootstrapped 100 times.

Detection Limit of *P. salmonis* by PCR

Real Time PCR (TaqMan assay)

To determine the detection limit of the PCR assay, a quantitative real-time TaqMan assay was developed. The assay was performed using dilution samples of DNA extracted from LF-89 isolate grown and titrated in cell culture.

An ABI Prism®7700 Sequence Detection System and software Sequence Detector version 1.9 (PE Applied Biosystems) were used for the analysis and storage of data. Primers and probe for the multiplex TaqMan assay were designed using Primer Express Software version 1.5 (PE Applied Biosystems). The *P. salmonis* primers and probe were based on the 23S rDNA gene, a relatively conserved genomic region among several isolates of *P. salmonis*. Primer and probe sequences were as follows: Forward primer (F-760): 5'-tctgggaagtgtggcgataga-3'; reverse primer (R-836): 5'-tcccgacctactcttgtttcatc-3'; 6-carboxyfluorescein (FAM) and 6-carboxytetramethylrhodamine (TAMRA), labelled probe (PS23S): 6FAM-tgatagccccgtacacgaaacggcata-TAMRA.

The 23S primers were used at a final concentration of 900nM. The 16S primers were used at a final concentration of 113nM. The 23S FAM probe and the 16S VIC probes were used at a final concentration of 250nM and 31nM, respectively. The reactions were carried out in a 96-well plate in a 25µL reaction volume containing 12.5µL Universal Master Mix (PE Applied Biosystems). Aliquots (2µL) of each DNA sample were added. Standard thermal cycling conditions were used (50^{O}C for 2 min, 95^{O}C for 10 min, followed by 40 cycles of 95^{o}C for 15 sec and 60^{O}C for 1 min). All reactions were repeated three times independently to ensure the reproducibility of the results. A sample was considered positive when the change in fluorescence (ΔR_n) of FAM or VIC, relative to that of ROX (internal reference signal), exceeded the set threshold values of 0.055 for FAM and 0.045 for VIC in the linear range of the amplification plots at a cycle threshold (C_T) value below 40. C_T is defined as the cycle at which a statistically significant increase in fluorescence output above background is detected. In addition, two generic 16S rDNA probes were used as endogenous controls to validate the DNA extraction procedure and to confirm the presence of bacterial DNA in negative control wells. The 16S rDNA assays were multiplexed with the *P. salmonis* test and therefore were performed under primer limiting conditions. The set of 16S primers and probe was based on a highly conserved region of the 16S rDNA region of bacteria (Gurtler and Stanisich, 1996) and had the following sequences:
Forward primer (F-1299): 5'-ctccatgaagtcggaatcgc-3'
Reverse primer (R-1399): 5'-aacccactcccatggtgtga-3'
Probe (generic 16S) VIC labelled: 6VIC-ccgggccttgtacacaccgcc-TAMRA.

Molecular Probe for Hybridisation Use on Tissue Sections

Introduction

A simple and effective filter hybridization method to detect DNA from a pathogen is to spot crude or purified mixtures of nucleic acids directly onto the surface of a membrane, such as nylon or nitrocellulose, where they are immobilised by baking or U.V. irradiation. Dot blot procedures are rapid, semi-quantitative and can be used to detect the presence of specific nucleic acid in many samples simultaneously on the same filter (Keller and Manak, 1993).

Preparing nucleic acid probes with a stable non-radioactive Digoxigenin (DIG) label provides sensitivity without the problems associated with the use of radioactive probes. The DIG labelling method is based on a steroid isolated from digitalis plants. As the blossoms and the leaves of these plants are the only natural source of DIG, the anti-DIG antibody does not bind to other biological material. DIG is linked to the C-5 position of uridine nucleotides. The DIG-labelled nucleotides may be incorporated into nucleic acid probes by DNA polymerases. Hybridized DIG-labelled probes may be detected with high affinity anti-DIG antibodies that are conjugated to alkaline phosphatase or other enzymes or fluorophores. Detection is performed via colorimetric or fluorescence assays. The sensitivity of the detection reaction is routinely 0.1pg (on a southern blot) (Boehringer Mannheim Application Manual).

The following technique describes the use of a probe specific to the 23S rDNA operon of *P. salmonis*.

Equipment List

Hybridization oven or alternatively a heating block
Ultra violet light
Hybridization bags (Roche cat # 1666649) or small temperature resistant, sealable plastic boxes
Nylon membranes positively charged (Roche cat # 1417240)

Reagents (Roche)

DIG Oligonucleotide Tailing Kit (cat # 1417231)
DIG Nucleic Acid Detection Kit (cat # 1175041)
DIG wash and block buffer set (cat # 1585762)
DIG Easy Hybridization solution (cat # 1603558)
Poly (dA) (cat # 223581)
Poly (A) (vial #11 in -DIG Oligonucleotide Tailing Kit or cat # 108626)
Anti-DIG AP conjugate (antibody solution) (cat # 1093274)
NBT/BCIP stock solution (colour substrate)(vial 4 from DIG Nucleic Acid Detection Kit or cat # 1681451)

Buffers and Solutions

DIG Easy Hybridization solution (c.f. reagents section) containing 0.1mg/mL of poly (A) and 5mg/mLpoly d(A).

Probe-DIG easy hybridization solution:
8mL probe (~50pmol) into 2mL DIG Easy Hybridization solution containing 0.1mg/mL poly (A) and 5mg/mL poly d(A).

Washing buffer: from DIG wash and block buffer set (cat # 1585762) (10X stock) or 0.1M maleic acid, 0.15M NaCl; pH 7.5 ($20^{O}C$); 0.3% (v/v) Tween 20.

Maleic acid buffer: from DIG wash and block buffer set (cat # 1585762) (10X stock) or 0.1M maleic acid, 0.15M NaCl; adjust with NaOH (solid) to pH 7.5 ($20^{o}C$).

Detection buffer: from DIG wash and block buffer set (cat # 1585762) (10X stock) or 0.1M Tris-HCl, 0.1M NaCl, pH 9.5 ($20^{O}C$).

Colour substrate solution: 200mL NBT/BCIP stock solution in 10mL 1X detection buffer).

20X SSC (stock): Dissolve 175.3g NaCl and 88.2g sodium citrate in 800mL of H_20. Adjust the pH to 7 with few drops of a 10N solution of NaOH. Adjust the volume to 1L with H_20. Dispense into aliquots and sterilize by autoclaving.

10% SDS: Dissolve 100g of electrophoresis-grade SDS in 900mL of H_20. Heat to $68^{O}C$ to dissolve. Adjust the pH to 7.2 by adding few drops of concentrated HCl. Adjust the volume to 1L with H_20. Dispense into aliquot.

Quality Control

For the dot blot DNA hybridization assay, *P. salmonis* (positive control) and uninfected fish DNA or uninfected cell culture DNA (negative control) must be blotted on the same membrane used for the unknown samples. Control DNA for diagnostic tests are stored at $-20^{O}C$ until use and check-tested on a regular basis.

Procedures

Sample preparation

Sample preparation was as described in previous PCR procedures.

Specific probe design and production

The synthetic oligonucleotide PS-F-760 (23S rRNA operon) (sequence:5'-TCT GGG AAG TGT GGC GAT AGA-'3) is used as a probe as it specifically anneals to the DNA of exotic isolates of *P. salmonis* as well as to the DNA of the Tasmania RLO.

Labelling of probe using DIG Oligonucleotide Tailing Kit (Roche, cat # 1 417 231)

Add 100 pmol of oligonucleotide to sterile water to a final volume of 9mL (specific probe)(tube A).
To a different tube (tube B), add 5mL of control oligonucleotide (vial #6) and 4mL sterile H_2O (non-specific probe).
Add the following reagents to tube A and B (on ice):
4mL reaction buffer (vial 1)
4mL $CoCl_2$ (vial 2)
1mL DIG dUTP solution (vial 3)
1mL dATP solution (vial 4)
1mL 50 Units terminal transferase (vial 5)

Mix and centrifuge briefly.
Incubate at 37^{O}C for 15 min then put on ice.
Stop reaction by adding 2mL of 0.2M EDTA (pH 8.0).

Membrane blotting

Denature fish DNA samples (as well as the positive and negative controls) at 95^{o}C on a heating block for 5 min.
Put tubes on ice for 5 min.
Put 2mL of each sample on a labelled nylon membranes positively charged. (N.B. a second membrane receives the same DNA samples and is hybridized with the non-specific probe e.g. tube B).
Expose membranes to U.V. light for 10 min.
Incubate membranes for 30 min in 5mL of preheated hybridization solution at 60^{O}C (DIG Easy Hybridization solution).
Transfer membrane to two different hybridization bags containing 2mL of probe specific (2mL of probe in 2mL of preheated hybridization solution) and probe non-specific (2mL of probe in 2mL of preheated hybridization solution) preheated hybridization solution at 60^{O}C.
Incubate membranes for 30 min at 60^{O}C (with occasional mixing of solutions).
Wash membranes twice 5 min in ample 2X SSC; 0.1% SDS solution at room temperature.
Wash sections twice 15 min in 0.5X SSC; 0.1% SDS at hybridization temperature (55^{O}C).

Immunological detection of hybridized probe

Rinse sections briefly in 1X washing buffer (DIG wash and block buffer set cat # 1585762).
Incubate membranes in 10mL of 1X blocking solution (DIG wash and block buffer set cat # 1585762) for 30 min at room temperature.
Tip out blocking solution and add 10mL of new blocking solution containing 2mL of anti-DIG antibody solution.
Incubate membranes for 30 min at room temperature (with occasional mixing of solutions).
Wash twice 15 min. with ample volume of washing buffer 1X.

Equilibrate 5 min. in 1X detection buffer (DIG wash and block buffer set cat # 1585762).
Add membranes in 10mL of colour substrate solution (200μL NBT/BCIP stock solution in 10mL of 1X detection buffer).
Incubate in dark for 10 to 30 min.
Stop reaction with deionised H_2O.

Interpretation

Positive reaction: Focal, purple-blue staining indicates presence of *P. salmonis* DNA and Tasmanian RLO DNA.

Negative reaction: No blue staining apparent. Non-specific probe does not show blue staining.

Background staining**:** Pale, bluish staining may occur and could be due to low level of non-specific probe hybridization or non-specific binding of antibodies rather than non-specific binding of probe (Johnson *et al*., 1984).

RESULTS AND DISCUSSION

Importation of Reference Materials

Exotic strains of *Piscirickettsia salmonis*

Actively growing cultures of reference strains of *Piscirickettsia salmonis* (Table 2) were imported from the OIE Reference Laboratory (Dr J. L. Fryer, Oregon State University, Corvallis, Oregon 97331-3804 USA). On arrival at AAHL, the cultures were examined, by light microscopy, for the appearance of CPE. While CPE had developed to a certain degree, it was not fully developed in all cultures. Thus the cultures were incubated further at 15^{O}C until CPE was complete (designated 100% CPE i.e. all host cells destroyed). Following complete destruction of the cell monolayers, replicate aliquots of the cell culture supernatants were either stored at 4^{O}C, cryopreserved in liquid nitrogen, or passaged onto fresh cell cultures for further study.

Table 2. Imported reference strains of *Piscirickettsia salmonis*

Reference Strain	Country of Origin	Reference
LF-89	Chile	Fryer *et al*. 1992; House *et al*. 1999; OIE 2003
ATL-4-91	Canada	House *et al*. 1999; Mauel *et al*. 1999
NOR-92	Norway	Olsen *et al*. 1997; House *et al*. 1999; Mauel *et al*. 1999

Tasmanian Rickettsia-like Organism (RLO)

As part of this project, the Tasmanian RLO was incorporated into the research and development activities and served several purposes. Firstly, the use of the Tasmanian RLO assisted in determining whether the established procedures were pan-specific for RLOs or whether they were *P. salmonis*-specific. In addition, preliminary characterisation of the Tasmanian RLO, undertaken as part of this project, assisted in determining those characteristics which appeared to be *P. salmonis*-specific.

Moreover, in obtaining samples from the field, difficulties in detection and identification of RLOs were highlighted, and demonstrated those areas in which greater emphasis should be placed. The submissions analysed during the course of this project are shown in Appendix 4.

Innocuity and Purity Testing

PCR Analysis

The identity of the 3 exotic strains of *P. salmonis* was demonstrated by PCR analysis. Firstly, bacterial genomic DNA was extracted from lysed cell culture supernatant derived from the imported cultures, using the QIAamp DNA mini kit. Then, a nested PCR, using general bacterial 16S rDNA primers in the first amplification reaction and *P. salmonis* specific primers in the second reaction

(OIE, 2003), was performed on the extracted DNA. A gene fragment of the expected molecular mass was obtained for the 3 strains samples (LF-89, ATL-4-91, NOR-92) as well as the Tasmanian RLO (Figure 1). No amplified product was obtained from DNA extracted from unrelated fish bacteria (used as negative controls in the assay) confirming the specificity of the assay.

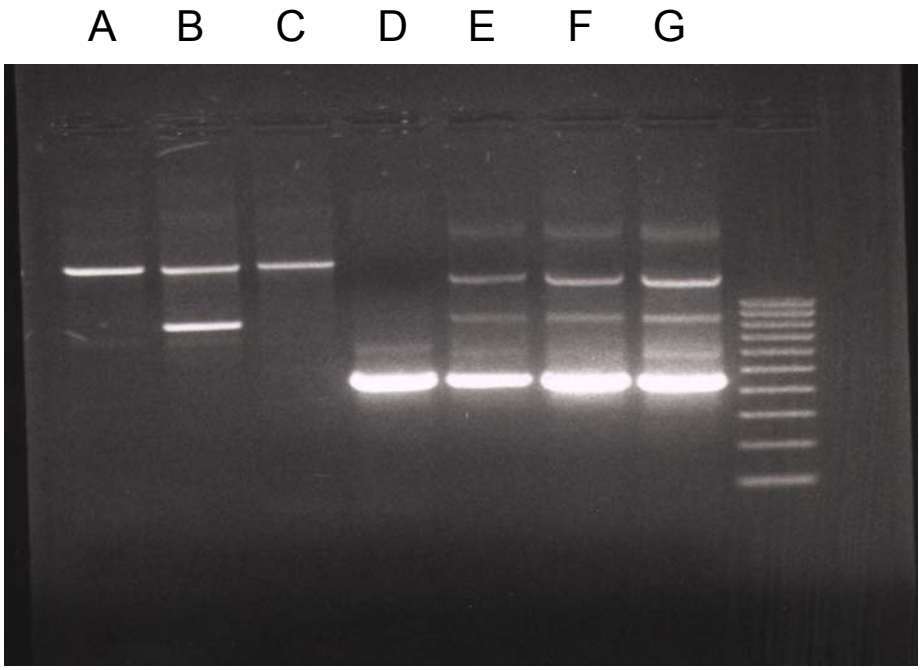

Figure 1. Photograph of ethidium bromide-stained amplicons obtained from the nested *P. salmonis* PCR. The *P. salmonis*-specific nested PCR amplicons (bright bands) of 469 base pairs in size are seen in wells D, E, F and G. Wells E, F, and G contained DNA samples extracted from supernatants of cell cultures infected with the exotic *P. salmonis* isolates LF-89, ATL-4-91, and NOR-92, respectively. Well D contained a DNA sample extracted from fish tissue infected with the Tasmanian Rickettsia-like organism (RLO). Wells A, B, and C contained DNA samples from unrelated fish bacteria used as negative controls. The last well on the right-hand-side of the gel contained the 100 bp ladder. Note that in addition to the specific bands in lanes D, E, F and G, there are some non-specific bands in each lane, including lanes D, E, F and G.

All *Piscirickettsia* samples, including the Tasmanian RLO, yielded specific bands of the correct size. Moreover, sequence analysis of the products form the *P. salmonis* isolates confirmed that the PCR products were specific for *P. salmonis*. In contrast, sequence analysis showed that the PCR product from the Tasmanian RLO was different but closely related to *P. salmonis*. Based on these PCR results it is confirmed that the imported reference strains do contain *P. salmonis*.

In order to confirm that the *P. salmonis* cell cultures were free from any contamination with IPNV, IHNV or VHSV, cell culture samples were processed with the QIAamp viral RNA mini kit. A RT-PCR was then performed on all cell

extract RNA according to the protocols currently used in our laboratory. All three *P. salmonis* cell culture samples were negative for this assay. The IPN, IHN and VHS viruses used as positive controls showed amplified gene fragments of expected molecular mass (results not shown), indicating that the assay was valid.

Bacteriological Culture

In order to confirm that the *P. salmonis* cultures were not contaminated with any other fish bacteria, 25µL of each cell culture supernatants were streaked on blood agar plates and incubated at room temperature for 4 days. No bacterial growth was observed indicating that the *P. salmonis* cell cultures were not contaminated.

Conclusions

Each of the *P. salmonis* reference strains was confirmed to contain *P. salmonis* with no other contaminating bacterial species nor any of the significant viral pathogens that could contaminate the CHSE-214 host cell cultures.

Cell Culture System for the Isolation and Growth of *P. salmonis*

Fish Cell Line Susceptibility

Five standard fish cell lines, CHSE-214, FHM, EPC, RTG-2, BF-2, were tested for their susceptibility to infection by the three reference strains of *P. salmonis*. All five fish cell lines tested were susceptible to infection. The ATL-4-91 and NOR-92 strains induced cytopathic effect (CPE) after approximately 7 days post-inoculation. About 90% of the cell monolayer was destroyed by day 20 post-inoculation. The LF-89 strain induced CPE after approximately 15 days post-inoculation. About 90% of the cell monolayer was destroyed by day 35 post-inoculation. An example of *P. salmonis*-induced CPE is shown in Figure 2.

Of interest, none of the submissions from Tasmania that were processed for RLO isolation in cell culture yielded positive results. Our inability to grow the Tasmanian RLO in cell culture could be/was due to any one, or more, of the following reasons:

1. Submitted samples were contaminated with other bacteria which destroyed our cell cultures;

2. Submitted samples contained Atlantic salmon reovirus which destroyed our cell cultures;

3. Submitted samples did not contain viable RLOs;

4. The Tasmanian RLO will not grow in our cell culture system due to some specific cultural needs.

Since the OIE recommends against the use of antibiotics for *P. salmonis* isolation, bacterial contamination was a problem initially. Some experiments (see below) were carried out to determine whether any of the more common antibiotics could be of use in *P*. salmonis isolation in cell culture.

In addition, it was shown, for some of the submissions from Tasmania, that Atlantic salmon reovirus was isolated in cell culture (results not shown). It is possible that RLO was also present but the viral infection destroyed the host cells before the RLO could develop. A rabbit polyclonal antiserum specific for the Atlantic salmon reovirus is available at AFDL and its ability to neutralise the reovirus was examined. Unfortunately the antiserum was found to be non-neutralising and therefore would not be of use in RLO isolation from samples containing Atlantic salmon reovirus.

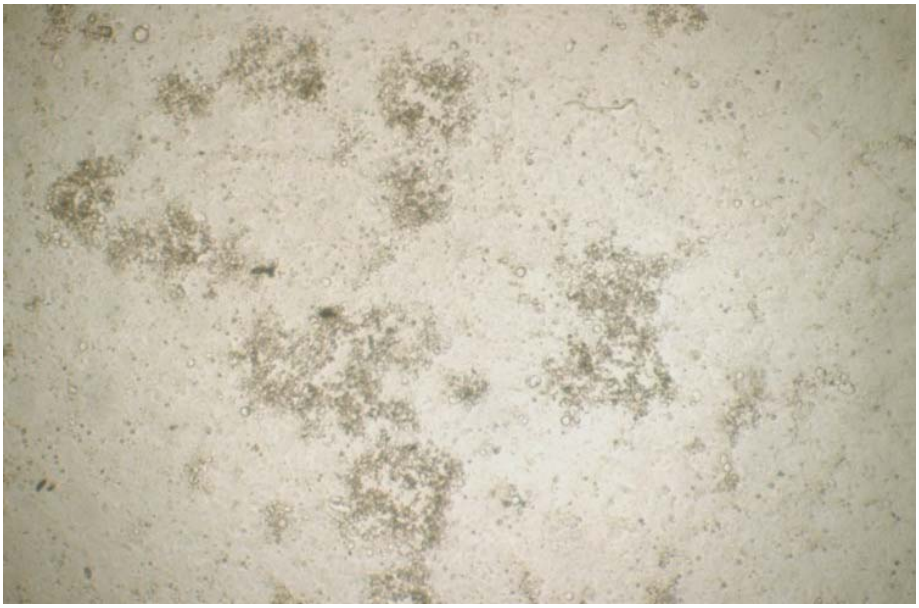

Figure 2. Photomicrograph of CPE, typical of *P. salmonis*, developing in cell cultures of CHSE-214 cell line. Magnification 40X, CHSE-214 cultures at day 11 post-infection with *P. salmonis* NOR-92 strain (Norway) in the presence of 100µg/mL ampicillin. 100µL of a 10^4 $TCID_{50}$/mL stock suspension of *P. salmonis* was used to infect cells.

To determine whether samples submitted from Tasmania contained viable RLO, samples were processed to provide inocula for Atlantic salmon in infectivity trials (see below).

Experimental Infections

One experimental infection experiment was undertaken, over a period of 30 days. All fish were monitored daily for clinical signs. No negative control (inoculated with medium only) fish died or showed any clinical signs over the course of the experiment. In contrast, at day 11 post-inoculation (p.i.), 1 fish was found dead and two fish were moribund in the experimentally infected group. In addition, some fish showed clinical signs of disease such as reddening of the eyes and reddening of the skin at the base of the fins.

During the course of the experiment all dead and moribund fish were removed from tanks. Moribund fish were kept on ice and processed in the laboratory for tissue culture growth of *P. salmonis*. Some of the livers harvested showed white spots.

In total, during the course of the experiment, 14 fish were found dead and 5 moribund fish were observed in the experimentally infected group and were harvested for tissue culture, PCR analysis and tissues were also fixed for histopathology, immunohistochemistry, electron microscopy (Figure 3) and immuno-electron microscopy (Figure 4). No control fish died.

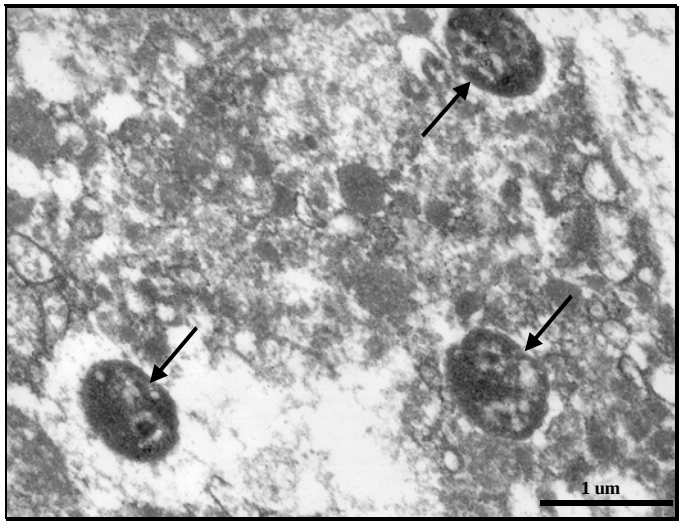

Figure 3. Electron photomicrograph of an ultrathin section of Atlantic salmon tissue infected with the Tasmanian Rickettsia-like organism (RLO). The arrows point to three individual intracellular organisms. Magnification 35 000X.

Assuming that the moribund fish would have died within 24 hrs if not harvested, a total of 54% of the fish injected with the RLO suspension died within 25 days.

RLO PCR assay performed on some of the dead and moribund fish yielded positive results for RLO indicating that the Tasmanian RLO was responsible for the fish mortality (see Appendix 4 for a list of submissions to AAHL). These results also demonstrate that RLO present in fish tissues collected from sick, farmed Atlantic salmon and transported on ice from Tasmania to AAHL remained viable. Thus the inability of the RLO to grow in cell culture was not due to a loss of viability during transportation and processing.

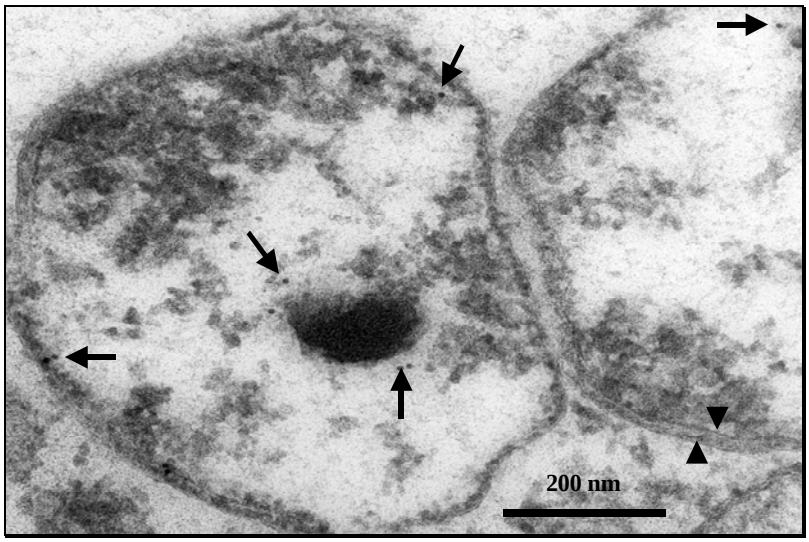

Figure 4. Immuno-electron photomicrograph of Atlantic salmon tissue infected with the Tasmanian RLO. Ultrathin sections were incubated with anti-*P. salmonis* LF-89 isolate antiserum (diluted 1:200 in PBS) for 2 h at 24^{O}C, washed with PBS, incubated with protein A-gold (diluted 1:100) at 24^{O}C, washed, post-fixed with 2.5% cacodylate-buffered glutaraldehyde, rinsed and stained with lead citrate and uracyl acetate. Specimens were examined at either 75 kV or 100 kV with a Hitachi H7000 scanning transmission electron microscope, calibrated with a 2160 line/mm grating replica. Note the specific gold bead labelling (arrows) and the bi-layer undulating membranes (arrow heads) of the RLO. Magnification 45 000 X.

Optimisation of the Cell Culture System

Quantification of *P. salmonis* Growth in Cell Culture

To determine the most appropriate cell line to be used for *P. salmonis* isolation and growth, titration experiments were carried out in each of the five fish cell lines in common use at AAHL. These cell lines included those recommended by the OIE (OIE, 2003). Based on the titration results in the different fish cell lines, the cell line of choice is CHSE-214 and the back-up cell line is BF-2 which is in contrast to OIE recommendations (CHSE-214 and EPC as the preferred cell lines). Although all cell lines tested were susceptible to infection, CHSE-214 and BF-2 cell lines are preferred due to ease of handling.

Culture medium used was Eagle's MEM supplemented with 10% (v/v) foetal bovine serum and 2mM L-glutamine. To date, the Tasmanian RLO has not been isolated in cell culture.

Use of Antibiotics

In order to reduce the negative impact of any contaminating bacteria in submitted samples, a range of antibiotics and antimycotics were evaluated for their application in *P. salmonis* isolation (Table 3).

Preliminary studies indicate that the exotic strains of *P. salmonis* can grow in CHSE-214 cultures ($15^{O}C$) in the presence of the following antibiotics/antimycotics:

-Ampicillin (100µg/mL)
-Penicillin (100µg/mL)
-Fungizone (2.5µg/mL)

Table 3. Determination of *P. salmonis* susceptibility to various antibiotics in cell culture

Antibiotics (Sigma Cat. #)	CPE	Final conc. (µg/mL)	Targets	Resistance/ susceptibility
Medium only	++++	n.a.	n.a.	n.a.
Erythromycin (Cat # E-5389)	+	100	Gram +/-	Susceptible
Kanamycin monosulphate (Cat # K-1377)	++	100	Gram +/-, mycoplasma	Partially susceptible
Streptomycin sulphate (Cat # S-9137)	++	100	Gram +/-	Partially susceptible
Neomycin sulphate (Cat # N-6386)	++	50	Gram +/-	Partially susceptible
Ampicillin (Cat # A-0166)	++++	100	Gram +/-	Resistant
Gentamicin sulphate (Cat # G-1264)	+	50	Gram +/-, mycoplasma	Susceptible
Penicillin G (In house source)	++++	100 U/mL	Gram +	Resistant
Fungizone (Gibco, Cat # 15295-017)	++++	2.5	Fungi	Resistant
Amp/Pen	+++	100/100	Gram +/-	Partially susceptible

Purification of Cell Culture-derived *P. salmonis* for Polyclonal Antiserum Production

Since a commercial polyclonal antiserum became available during the course of this project it was decided that, rather than produce another polyclonal antiserum, the commercial antiserum should be evaluated as a diagnostic reagent for use in Australian laboratories (see below).

Polyclonal Antiserum against the Tasmanian RLO

To date, the Tasmanian RLO has not been isolated in cell culture, therefore the RLO could not be purified easily and a specific antiserum could not be produced within the time-line and budget of this project. The use of subtractive hybridisation in an attempt to identify gene sequences of potential use in the production of a diagnostic antiserum was investigated.

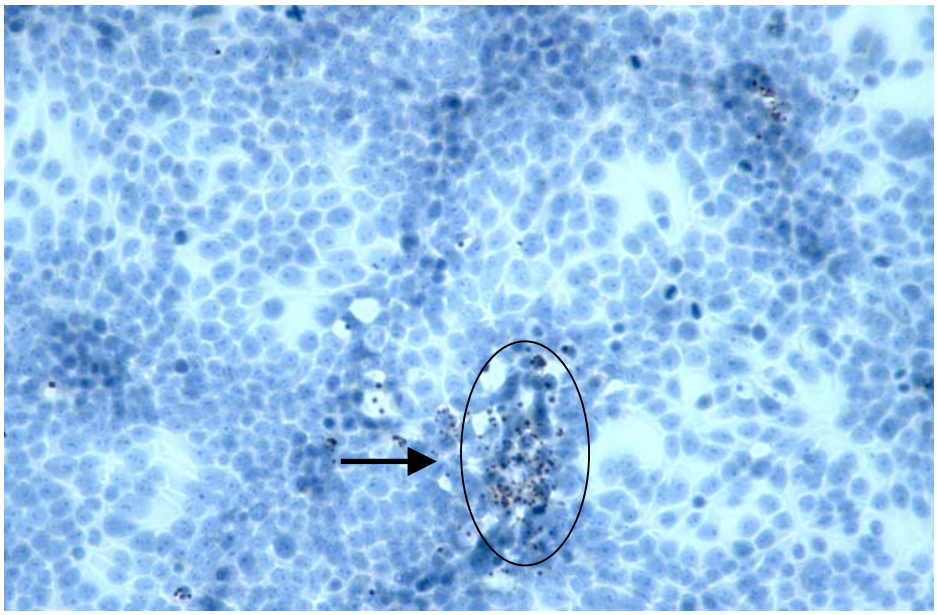

Figure 5. Photomicrograph of *P. salmonis* (ATL-4-91 isolate)-infected CHSE-214 cell monolayer. The bacterial cells (black dots) are visible by light microscopy. The cytopathic effect (CPE) on the cell monolayer is not yet visible at this stage of the infection. The bacterial cells are identified as *P. salmonis* by the pale pink colour produced in the immunoperoxidase assay with the specific primary antibody.

Subtractive Hybridisation

To date, a dozen clones of tester-specific DNA fragments have been sequenced. One third of the clones matched fish gene sequences in the Gen Bank database (background clones not related to the RLO). The remaining clone sequences showed sequence homologies with various organisms including bacteria.

Time has not allowed further work in this area to be undertaken during the course of this project. Further work, depending on resources, will include:

- The sequencing of more clones
- The synthesis of probes based on the clone sequences
- Dot blots hybridization using DNA samples extracted from:
  a) *P. salmonis* grown in cell culture,
  b) Uninfected cell culture supernatant,
  c) RLO infected fish tissues
  d) Uninfected fish tissues,
  e) Unrelated bacteria.

The dot blot analysis will indicate whether the clones do contain RLO-specific genes.

Identification of *P. salmonis* and other RLOs by Immunoperoxidase Assays

Detection of *P. salmonis* in Cell Cultures by Immunocytochemistry

An immunoperoxidase test, using the commercial polyclonal antiserum as the primary antibody specific for *P. salmonis*, was developed for the detection and identification of *P. salmonis* in fish cell cultures. The laboratory procedures are detailed in a Standard Diagnostic Test documented for Australian laboratories (Appendix 3). Results indicated that *P. salmonis* can be detected in cell culture (Figure 5).

Because the Tasmanian RLO cannot be grown in cell culture, it could not be determined whether this antiserum could be used to detect the RLO *in vitro*.

Detection of *P. salmonis* in Fixed Fish Tissues by Immunohistochemistry

In addition to detection and identification of *P. salmonis* in cell cultures, the ability of the commercial polyclonal antiserum to detect *Piscirickettsia* in fish tissues was also evaluated. Paraffin blocks of fixed infected fish tissues, received from the USA, were used to validate the immunoperoxidase assay in fish tissues. Tissue sections cut from this block were processed for a standard immunoperoxidase test using the commercial polyclonal antiserum as the primary antibody (see Appendix 3 for details). Figure 6 demonstrates the specific staining using this test and the localisation of *P. salmonis* within tissues of infected fish.

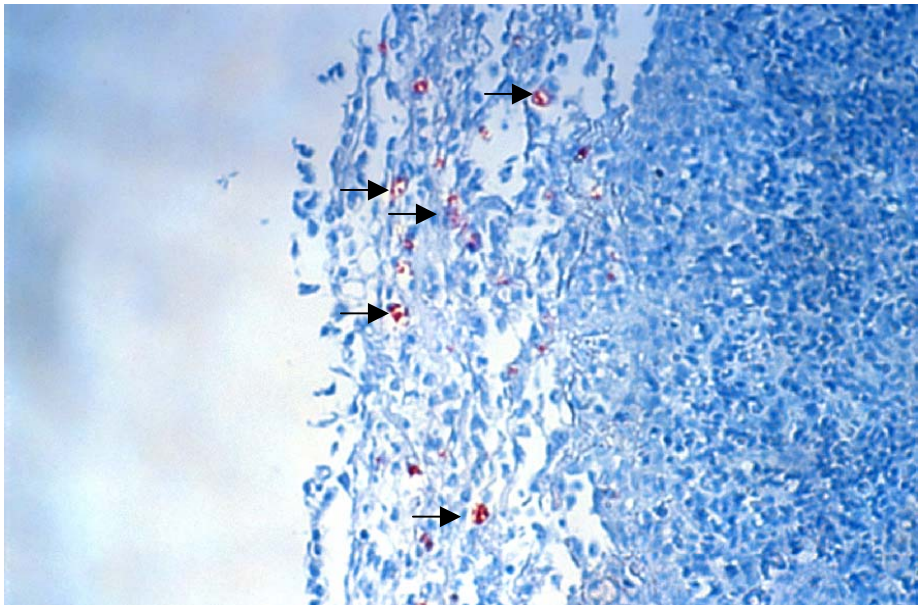

Figure 6. **Photomicrograph of fish tissue fixed 7days after infection with the LF-89 isolate of *P. salmonis*.** The immunoperoxidase assay was carried out using the polyclonal antiserum directed against the LF-89 isolate of *P. salmonis*. The bright red staining (some examples are arrowed) demonstrates the presence of *P. salmonis* bacteria.

In this test the *P. salmonis* is localised in the fish tissue by the intense red staining produced by the immunoperoxidase-catalysed reaction.

In order to determine whether the immunoperoxidase assay could identify the Tasmanian RLO in fish tissues, Atlantic salmon were inoculated with the Tasmanian RLO strain and sacrificed at various times post-inoculation for laboratory investigation. Tissues from internal organs were obtained, fixed and the immunoperoxidase assay was performed on tissues sections. Results indicated that the RLO can be detected in infected tissues (Figure 7).

However, the weaker staining observed suggested that the Tasmanian RLO antigenic determinants differ from those of *P. salmonis* isolates. In a similar study involving the isolation of a White Sea Bass 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.*

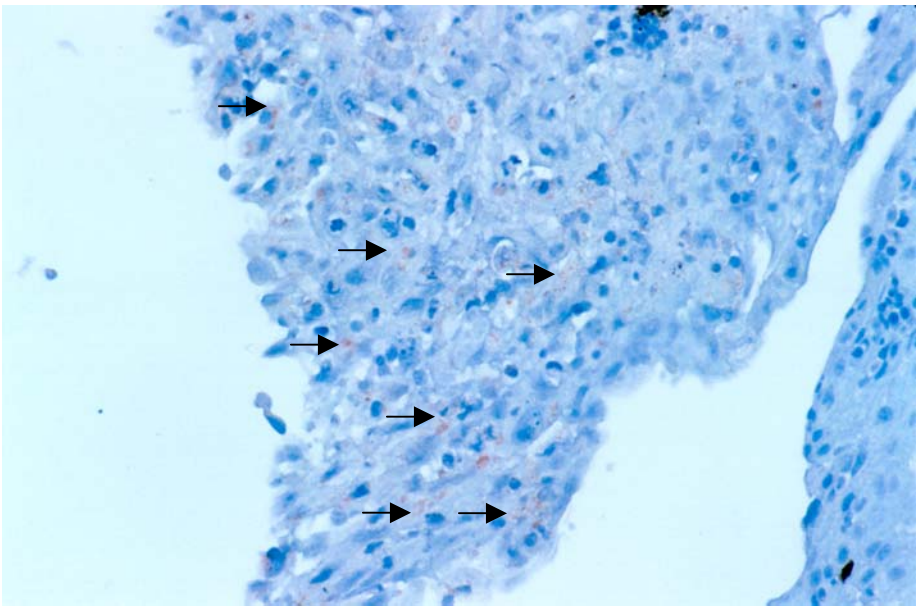

Figure 7. Photomicrograph of fish tissue fixed 13 days after infection with the Tasmanian RLO. The immunoperoxidase assay was carried out using the commercial polyclonal antiserum directed against the LF-89 isolate of *P. salmonis*. The pale red staining (some examples are arrowed) indicates the presence of RLO but note that the intensity of the staining is reduced compared with the results of immunoperoxidase assays undertaken on the homologous *P. salmonis* LF-89 isolate (Figure 6).

PCR Assay for the Identification and Differentiation of *P. salmonis* Strains

The inability of the Tasmanian RLO to grow in cell cultures susceptible to *P. salmonis* and the weak cross-reaction observed with the commercial polyclonal antiserum raised against *P. salmonis* indicate that there are significant differences between the exotic *P. salmonis* reference strains and the Tasmanian RLO. In addition, amplicons produced by the nested PCR indicated further differences at the nucleic acid level.

```
consensus    AGCTAGTTGGTAGGGTAAAGGCTTACCAAGGCGACGATCCATAGCTGGTTTGAGAGAATGGC
EM_90          ............................A.............................
ATL_4_91       ..................-...........................................
NOR_92         ...........................................................
LF_89          ...........................................................
SLGO_94        ...........................................................
RLO Tas        ...........................................................

consensus      CAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATAT
EM_90          ...........................................................
ATL_4_91       ...........................................................
NOR_92         ...........................................................
LF_89          ...........................................................
SLGO_94        ...........................................................
RLO Tasmania   ...........................................................

consensus      TGGACAATGGGGGGAACCCTGATCCAGCAATGCCACGTGTGTGAAGAAGGCCTTAGGGTT
EM_90          ............A...........C--..-...............................
ATL_4_91       ...........................................................
NOR_92         ...........................................................
LF_89          ...........................................................
SLGO_94        ...........................................................
RLO Tasmania   ...........................................................

consensus      GTAAAGCACTTTCAGCGGGGAGGA-AGGTAAGCTAATTAATACTTGGCTTAATTGACGTT
EM_90          .....-.....................................................
ATL_4_91       ........................G..................................
NOR_92         ...........................................................
LF_89          ...........................................................
SLGO_94        ...........................................................
RLO Tasmania   ............................G.CTGAT.C.......ATTGG.C.........

consensus      ACCTGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCG
EM_90          ....................................G..................C...
ATL_4_91       ......................-....................................
NOR_92         ...........................................................
LF_89          ...........................................................
SLGO_94        ...........................................................
RLO Tasmania   ...........................................................

consensus      AGCGTTAATCGGAATTACTGGGCGTAAAGGGCGCGTAGGCGGAAGATTAAGTTGGATGTG
EM_90          ...........................................................
ATL_4_91       ...........................................................
NOR_92         ...........................................................
LF_89          ...........................................................
SLGO_94        ...........................................................
RLO Tasmania   ...........................................................

consensus      AAATCCCAGGGCTCAACCTTGGAACTGCATCCGAAACTGGTATTCTAGAGTATGGTAGAG
EM_90          ...........................................................
ATL_4_91       ...........................................................
NOR_92         ...........A...............................................
LF_89          ...........................................................
SLGO_94        ...........................................................
RLO Tasmania   ........................................T..................

consensus      GAAAGTGGAATTTCAGGTGTAGCG
EM_90          ..............-........
ATL_4_91       ........................
NOR_92         ........................
LF_89          ........................
SLGO_94        ........................
RLO Tasmania   ........TT.CG..........A
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Figure 8. Partial 16S rDNA nucleotide sequence alignment of the *P. salmonis* isolates and the Tasmanian RLO. The differences between the various strains and the consensus sequence are as shown (- specifies a deletion). The differences for the Tasmanian RLO are also highlighted in yellow.

Differences between various RLOs, including *P. salmonis*, were investigated further by sequence analysis of PCR amplicons. At AAHL, during investigations of suspect exotic diseases, it is policy to confirm the identity of PCR amplicons by nucleic acid sequencing. Sequencing of PCR products can be either out-sourced or undertaken, as at AAHL, in house if a sequencer is available.

Nested PCR Assay for the Detection of *P. salmonis*

Samples for PCR analysis were prepared from cell cultures infected with imported *P. salmonis* strains, as well as fish tissues obtained from Atlantic salmon inoculated with the Tasmanian RLO strain. The PCR used was the nested PCR documented in the OIE Manual of Diagnostic Tests for Aquatic Animals (OIE, 2003). The PCR products are shown in Figure 1.

Primers specific for the 16S region produced amplicons of expected size (Mauel *et al*., 1996) for all samples tested (Figure 1). All *Piscirickettsia* samples, including the Tasmanian RLO, yielded specific bands of the correct size. Moreover, sequence analysis of the products from the *P. salmonis* isolates confirmed that the PCR products were specific for *P. salmonis*. In contrast, sequence analysis showed that the PCR product from the Tasmanian RLO was different but closely related to *P. salmonis* (Figure 8).

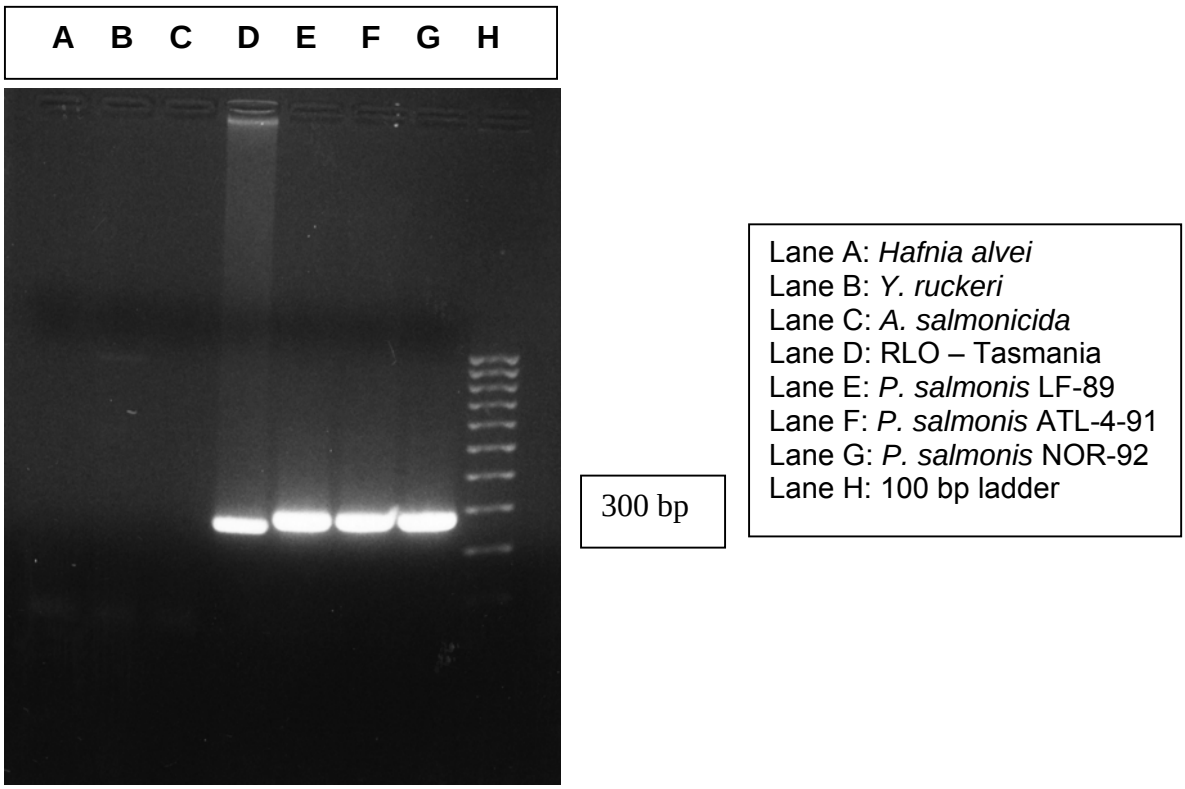

Figure 9. Single step PCR. The primers used for the rDNA amplification were RTS-1 and RTS-4 (Marshall *et al*. 1998), producing a 283 bp amplicon in *P. salmonis*. Several other species of bacteria were used as specificity controls. There is a suspicion that the Tasmanian RLO amplicon is slightly smaller than the *P. salmonis* amplicons.

Single step PCR for the Detection and Identification of *P. salmonis*

Nested PCR assays are not ideal as diagnostic tests due to the common problem concerning cross-contamination. While a nested PCR is of higher sensitivity than a single step PCR, a single step PCR may be sufficiently sensitive for detection and identification of *P. salmonis* in overtly diseased fish. Thus the use of a single step PCR was investigated for the detection and identification of *P. salmonis*.

By PCR analysis, using *P. salmonis*-specific primers (Marshall *et al*. 1998), each reference strain yielded a positive band of the correct size for *P. salmonis* (Figure 9). Sequence analysis confirmed that the PCR product was specific for *P. salmonis* (Figure 11).

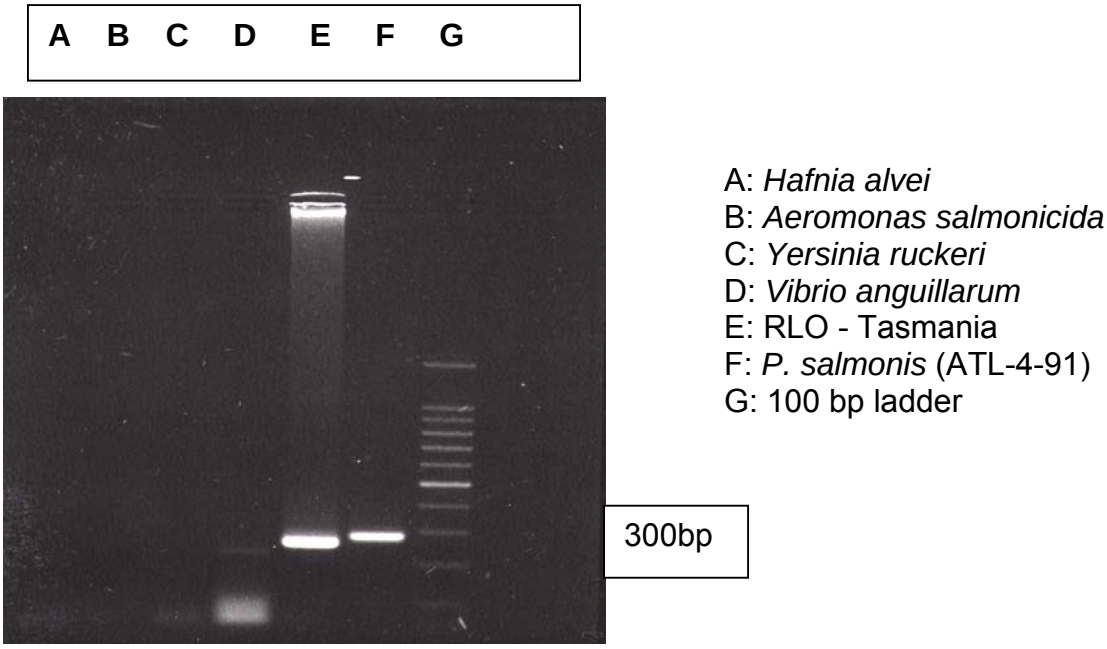

Figure 10. Detection of *Piscirickettsia salmonis* using a single-step PCR method. The primers used for the rDNA amplification were RTS-1 and RTS-4 (Marshall *et al*. 1998), producing a 283 bp amplicon in *P. salmonis*. Several other species of bacteria were used as specificity controls. *Vibrio anguillarum* shows a weak non-specific band just below the expected *P. salmonis* amplicon. The Tasmanian RLO strain has a band slightly lower than the Canadian strain (ATL-4-91). Sequencing revealed that the amplicon from the Tasmanian strain is shorter than the amplicon from exotic strains by 19 base pairs.

The RLO detected in Atlantic salmon from Tasmania also yielded a positive band indicating that the RLO was similar to *P. salmonis*. However, the band appeared to be of a slightly lower molecular weight than the band associated with the *P. salmonis* reference strains. Further experiments confirmed that the RLO amplicon (264 bp) was shorter than the *P. salmonis* amplicon (283 bp) by 19 base pairs (Figure 10).

Comparison of the Nucleic Acid Sequence of the Emerging Tasmanian RLO Isolates to Sequences of Known Exotic Strains of *P. salmonis*

Sequence Determination

The 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. Sequencing revealed a deletion of 19 consecutive base pairs located at the 3 prime-end of the ITS sequence which suggests a significant genetic divergence from *P. salmonis* (Figure 11).

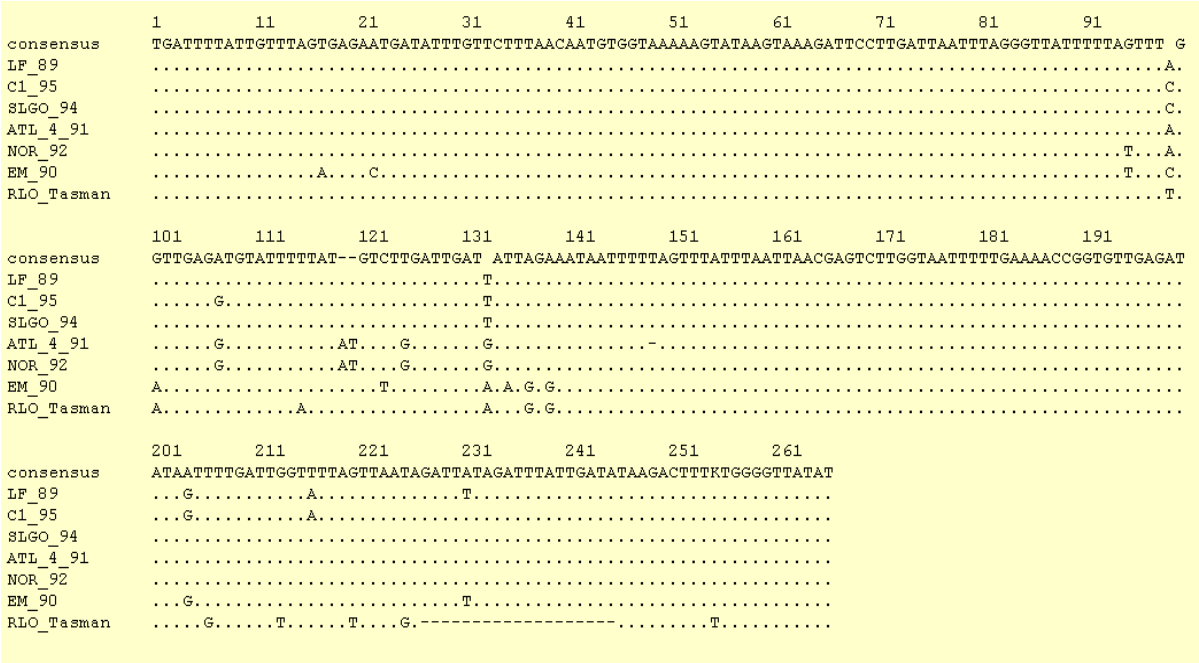

Figure 11. Partial ITS rDNA nucleotide sequence of the Tasmanian RLO. The RLO sequence shows a 19 base pair deletion at the 3'-end (- specifies a deletion) compared to the consensus sequence. Differences among other available RLO sequences are also shown.

Phylogenetic Analysis

Sequencing of the 16S (partial) and internal transcribed spacer (ITS) region of the rDNA operon of the Tasmanian RLO was performed. A nucleotide % similarity between the Tasmanian RLO and exotic isolates of *P. salmonis* was established. Results indicate that a fairly high % similarity exists between the Tasmanian RLO and the *P. salmonis* isolates for both 16S and ITS region of the rDNA operon (Table 4). The Tasmanian RLO sequences will be deposited in GenBank in the near future.

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 two sets of sequences, both trees suggested

that the EM-90 isolate of *P. salmonis* is most closely related to the Tasmanian RLO (Figures 12 and 13) of the isolates included in this study.

Table 4. Sequence similarity of the partial 16S rDNA (446 bases) and of the internal transcribed spacer (ITS) (265 bases) between the Tasmanian RLO and 6 isolates of *Piscirickettsia salmonis*.

% similarity of Tasmanian RLO to 16S/ITS sequence of reference isolate indicated					
LF-89	ATL-4-91	NOR-92	SLGO-94	EM-90	C1-95
95.7/88.64	94.8/86.80	95.5/88.35	95.7/88.64	93.5/89.0	n.a./87.36

n.a.: sequence data not available for C1-95 isolate.

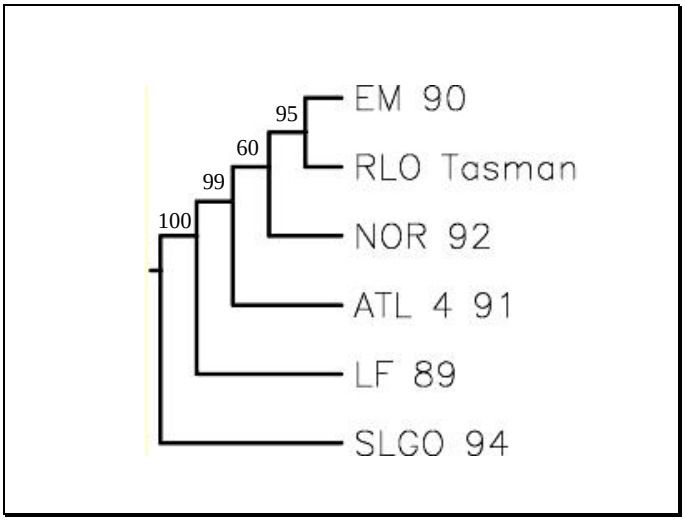

Figure 12. Phylogenetic tree: 16S rRNA operon. Tree designed using Angis: Phylogeny analysis package: Clustal W, Bootstrap, DNA distance (Jukes & Cantor), Neibor UPGMA, Consense. Bootstrap values are 100, 99, 60, 95%. Unrooted tree.

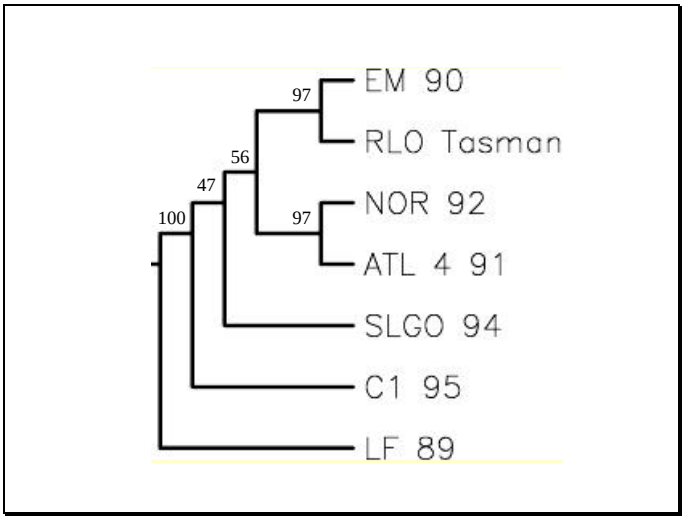

Figure 13. Phylogenetic tree: ITS region of the rRNA operon. Tree designed using Angis: Phylogeny analysis package: Clustal W, Bootstrap, DNA distance (Jukes-Cantor), Neibor (UPGMA), consense. Bootstrap values 100, 47, 56, 97 and 97%. Unrooted tree.

Conclusions

The Tasmanian RLO is related, but not identical, to the exotic *P. salmonis* isolates analysed in this project. Differences were noted in:

1. Ability to grow in cell culture
2. Reactivity with a polyclonal antiserum raised against one of the exotic *P. salmonis* strains (LF-89)
3. ITS nucleic acid sequence

Detection Limit of the *P. salmonis* PCR Assays

In order to quantify the detection limit of the *P. salmonis* PCR assays, a real-time PCR TaqMan assay was developed and performed using a dilution series of DNA extracted from the LF-89 isolate of *P. salmonis* titrated in cell culture. Results showed that the TaqMan PCR assay can detect *P. salmonis* DNA down to 0.5 $TCID_{50}$/mL (Figure 14). A full description of the *P. salmonis* TaqMan assay has been documented and published (Corbeil *et al*, 2003). In addition, the TaqMan assay is in current use as part of the Tasmanian Fish Health Surveillance Program.

To determine the detection limit as well as the sensitivity of the TaqMan assay, relative to the conventional nested PCR recommended by the OIE for the detection of *P. salmonis*, both assays were performed using the same dilution samples of DNA extracted from the isolate LF-89 titrated in cell culture. The conventional nested PCR showed that a specific band of an expected size of 476 base pairs was amplified for dilution samples down to the equivalent of 0.5 $TCID_{50}$/mL (Figure 10). This result is consistent with data obtained by Mauel *et al*.

(1996) who detected less than 1 $TCID_{50}$ using the conventional nested PCR assay. The TaqMan assay was shown to be equally sensitive as fluorescence was detected in all replicates down to a dilution equivalent to 0.5 $TCID_{50}$/mL with a mean C_T value of 36.37 (Table 5).

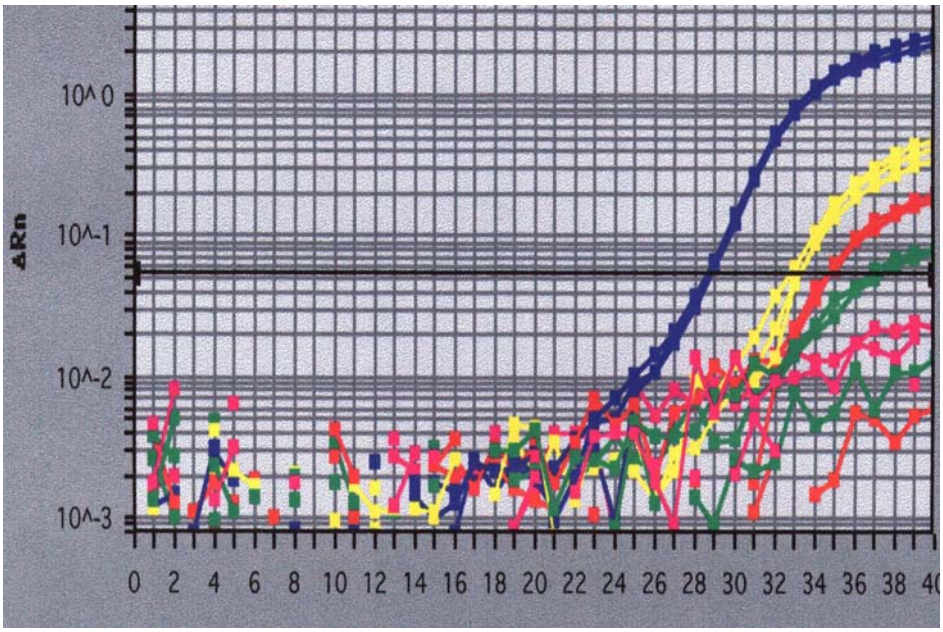

Figure 14. *P. salmonis* TaqMan assay. The graph shows the log of the change in fluorescence (y-axis) intensity versus the number of threshold cycles (x-axis) for each of several different target concentrations equivalent to the following Tissue Culture Infectious $Doses_{50}$/mL. Blue curve: 100 $TCID_{50}$/mL; yellow curve: 10 $TCID_{50}$/mL; red curve: 1 $TCID_{50}$/mL; green curve: 0.5 $TCID_{50}$/mL; pink curve: 0.1 $TCID_{50}$/mL. Note that for each sample preparation, the TaqMan assay is performed in triplicate (there are three curves for each target concentration). As the relative amount of target nucleic acid decreases, there is greater variation between the triplicates due to experimental error such as pipetting error. For example, one of green curves and one of the red curves are below the detection limit as would be expected for nucleic acid levels at the lower threshold of detection.

The TaqMan assay has the advantages that it is rapid, quantitative, and less prone to cross-contamination which can occur with conventional nested-PCR assays.

In addition, other non-piscine Rickettsia (*Neorickettsia helminthoeca*, *Rickettsia honei*, *R. prowazekii* and *Orientia tsutsugamushi kato*) were analysed using the TaqMan PCR. DNA from these non-piscine *Rickettsia* was not amplified in this assay demonstrating that the TaqMan assay appears to be specific for piscine *Rickettsia* detecting *P. salmonis* as well as the Tasmanian RLO (Corbeil *et al*., 2003).

Table 5. Results Summary of *P. salmonis* TaqMan assay demonstrating assay sensitivity

P. salmonis titre $TCID_{50}$/mL	Experiment 1 Average C_T value*	Experiment 2 Average C_T value	Experiment 3 Average C_T value	Mean	Standard Deviation
10^2	28.62	28.66	28.86	**28.71**	0.12
10^1	33.17	33.04	33.28	**33.16**	0.12
1	36.36	36.28#	35.05#	**35.89**	0.73
0.5	34.52#	37.00#	37.61#	**36.37**	1.63
0.1	40	40	40	**40**	0
CHSE-214	40	40	40	**40**	0

N.B. C_T values $\geq$ 40 indicate the absence of specific amplification. *Average of three values for each experiment. # One or two out of the three samples did not yield any product, suggesting that these dilutions approach the detection limit of the assay.

A similar series of dilutions were analysed by the nested PCR. Figure 15 demonstrates that the nested PCR and the TaqMan PCR have similar sensitivities, both assays being capable of detecting 0.5-1.0 $TCID_{50}$/mL DNA equivalents.

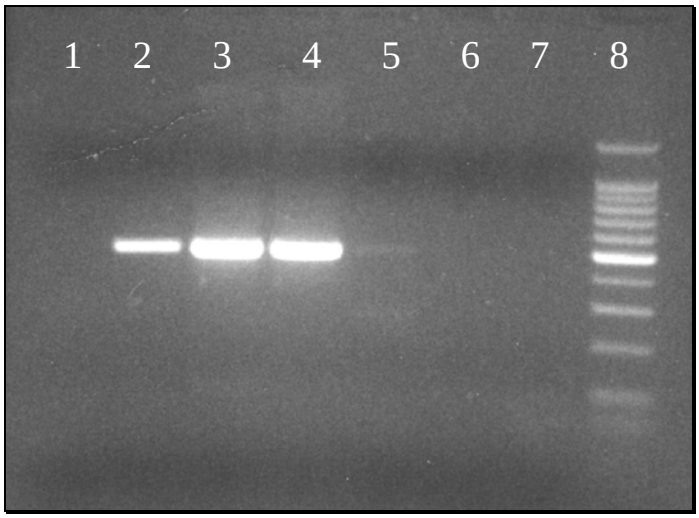

Figure 15. Photograph of a 2% agarose gel containing ethidium bromide stained amplicons demonstrating sensitivity of the *P. salmonis* nested PCR. Lane 1: Negative control CHSE-214 DNA; lanes 2-7 contain amplicons amplified from *P. salmonis* DNA diluted to 10^2 $TCID_{50}$/mL equivalents (lane 2), 10^1 $TCID_{50}$/mL equivalents (lane 3), 1 $TCID_{50}$/mL equivalents (lane 4), 0.5 $TCID_{50}$/mL equivalents (lane 5), 0.1 $TCID_{50}$/mL equivalents (lane 6), 0.01 $TCID_{50}$/mL equivalents (lane 7). Lane 8 contains the 100 bp molecular markers. Specific bands are visible in lanes 2-5. Also note that the bands in lanes 3 and 4 are larger than in lane 2 even though there is less specific DNA. By diluting the samples, non-target host DNA is reduced, making the PCR more efficient.

Conclusions

The various PCR assays developed here provide specific and sensitive diagnostic procedures for the detection and identification of *P. salmonis*, as well as the Tasmanian RLO. Sequence analysis of the PCR amplicons allows differentiation between the various isolates and the Tasmanian RLO. Based on the data collected to date, it is likely that these assays form the basis for a pan-specific procedure for the detection and identification of piscine RLOs, including *P. salmonis*.

Molecular Probe for *in situ* Hybridisation Use on Tissue Sections

The commercial, polyclonal sheep anti-*P. salmonis* antiserum reacts only weakly with the Tasmanian RLO and is unsuitable for localisation of RLO in infected fish tissues. Attempt were made to develop an *in situ* hybridisation probe based on the PCR amplicon sequence. While some initial success has been achieved, time has not allowed the full development of such a diagnostic reagent.

BENEFITS

Piscirickettsia salmonis is a pathogen of Salmonidae. Coho salmon (*Oncorhynchus kisutch*) appear to be the most susceptible species, but the disease has also been reported in farmed Atlantic (*Salmo salar*), chinook (*O. tshawytscha*), masu salmon (*O. masou*) and rainbow trout (*O. mykiss*). Juvenile pink salmon (*O. gorbuscha*) are also susceptible to infection. The disease has been described from Chile, Ireland, Norway, and both the West and East coasts of Canada (see Lannan *et al*., 1999 and OIE, 2003 for reviews).

Rickettsia-like organisms have been isolated from non-salmonid fish (Chen *et al*, 1994; Chen *et al*., 2000a; Chen *et al*., 2000b). The relationships of most of these organisms to *P. salmonis* is not fully understood, but the RLO isolated from white sea bass (*Atractoscion nobilis*) (Chen *et al*., 2000a), unlike the Tasmanian RLO, appears to be genetically and serologically indistinguishable from *P. salmonis*.

Thus it is clear that the major beneficiary is the salmonid aquaculture sector, the vast majority of which operates out of Tasmania. It has been shown that the RLO isolated from Atlantic salmon farmed in SE Tasmania is related to, but different from, *P. salmonis*. Whether the differences observed are sufficient to place the Tasmanian RLO in a different species to *P. salmonis* has not been determined. The diagnostic procedures established here permit the rapid detection and identification of *P. salmonis* and other RLOs which, in turn, provides the regulatory authorities and industry to make timely decisions on disease management.

The conventional PCR procedures can be transferred to all diagnostic laboratories with PCR capability. In addition, those laboratories with real-time PCR capability will also be able to establish and implement the TaqMan assay.

FURTHER DEVELOPMENT

While the immunohistochemical techniques established here provide additional tools for the detection and identification of *P. salmonis*, they are inadequate for use in the diagnosis of the Tasmanian RLO. Since the RLO cannot be grown in cell culture, production of a specific diagnostic antiserum has not been possible. Thus development of an *in situ* hybridisation probe based on the sequence of the PCR amplicons was initiated. However completion of this part of the work was beyond the scope of the current project. Nevertheless, development of an *in situ* hybridisation probe, specific for *P. salmonis* and/or other piscine RLOs, would provide a useful diagnostic tool for the confirmation of RLO association with histological lesions.

The inability of the RLO to grow in current cell culture systems severely limits progress on characterisation of this pathogen. All research is restricted to clinical samples and the inability to produce relatively large amounts of material in cell culture for antiserum production, molecular studies and biochemical analysis has not allowed a full comparison of the Tasmanian RLO with the exotic strains of *P. salmonis*.

PLANNED OUTCOMES

P. salmonis is a serious pathogen of salmonid fish species which can cause significant losses in farmed salmonids. The development/establishment of an internationally recognised Standard Diagnostic Technique for the detection and identification of *P. salmonis* and related pathogens will permit State/Commonwealth agencies to establish accurate information on the presence or absence of these pathogens in fish populations. Thus the use of these procedures will play an important role in the management of any disease outbreak caused by *P. salmonis* or other RLOs.

Thus the industry sectors which will benefit from these outcomes include salmonid aquaculture in Tasmania and other states. Identification of infected and uninfected stock will allow industry and state officials to implement a disease management plan based on accurate information. Isolation of infected and uninfected fish populations will be possible thus enhancing our capability to control and/or eradicate the disease.

State diagnostic laboratories will benefit from being provided with sensitive and specific reagents and procedures. Based on accurate diagnoses, State officials responsible for conducting activities of the Local Disease Control Centre (LDCC) will be able to make informed decisions during implementation of disease management procedures.

Piscirickettsiosis, caused by *P. salmonis*, is listed by the OIE and, as a Member Country, Australia is obliged to report on its presence or absence in Australia. Such reporting can only be achieved if there are sensitive and specific reagents and procedures in place which are recognised by international agencies. Using internationally accepted procedures Australia can be confident of providing accurate information to the OIE, other international agencies and to our international trading partners.

Sequence data comparing the Tasmanian isolate with other exotic isolates will assist in determining the relationship between the Tasmanian isolate and these exotic and pathogenic strains. Such data will be useful in identifying any new variants which may occur in the future as well as identifying any incursion of potentially highly pathogenic strains. Again, this type of information will allow appropriate management procedures to be put in place, as required.

CONCLUSION

The aim of this project was to establish a range of Standard Diagnostic Procedures for *Piscirickettsia salmonis*. In addition, the procedures were applied to the preliminary characterisation of the Tasmanian RLO, recently isolated from Atlantic salmon farmed in S.E. Tasmania.

Thus a standard cell culture system for the isolation and growth of *P. salmonis* has been introduced and optimised at AAHL. It is interesting to note that while samples of the Tasmanian RLO were shown to be viable by inoculation into susceptible fish, the RLO did not grow *in vitro*.

A single-step PCR assay and a nested PCR assay were established and optimised for the identification and differentiation of *P. salmonis* strains (exotic) and the RLO (enzootic) present in cell culture supernatant and in fish tissues. The specificity of the PCR assays was demonstrated by using a range of bacterial isolates as negative controls, including the piscine bacterial pathogens *Yersinia ruckeri*, *Aeromonas salmonicida* and *Vibrio anguillarum*. In addition, DNA from the fish pathogen *Hafnia alvei*, which demonstrates some genomic similarity to *P. salmonis*, was also included as a negative control. None of the negative controls yield *P. salmonis*-specific amplicons when amplified with the *P. salmonis* primers. PCR analysis of the Tasmanian RLO demonstrated that the RLO was related to, but different from, the exotic reference strains of *P. salmonis*. Comparison of the nucleic acid sequence of the emerging Tasmanian RLO to sequences of known exotic strains demonstrated significant differences.

During the course of this project a new technology (real-time PCR) became available and instrumentation was obtained at AAHL to allow the development of a TaqMan PCR for *P. salmonis*. This newer technology has several advantages over conventional PCR including a greatly reduced risk of cross-contamination, a capability to run in a microplate format as well as being quantitative. Determination of sensitivity is thus facilitated.

It was demonstrated that both the nested PCR and the TaqMan PCR had equivalent sensitivities detecting around 0.5-1.0 $TCID_{50}$/mL DNA equivalents. In addition, other non-piscine rickettsia (*Neorickettsia helminthoeca*, *Rickettsia honei*, *R. prowazekii* and *Orientia tsutsugamushi kato*) were analysed using the TaqMan PCR. DNA from these non-piscine *Rickettsia* was not amplified in this assay demonstrating that the TaqMan assay appears to be specific for piscine *Rickettsia* detecting *P. salmonis* as well as the Tasmanian RLO.

Immunoperoxidase assays, using a commercial polyclonal antiserum raised against one of the reference strains (LF-89), for the identification of *P. salmonis* in cell cultures and fish tissues were established. It was demonstrated that this antiserum was capable of localising the exotic reference strains of *P. salmonis* in fish tissues as well as in cell cultures. However, the detection of the Tasmanian RLO by this immunohistochemical assay was far less convincing, indicating that the epitopes recognised by the antiserum are either qualitatively or quantitatively different for the RLO.

Thus, as part of this project, it has been demonstrated that the Tasmanian RLO is similar to, but different from, *P. salmonis*. Differences have been demonstrated at the genotypic level (nucleic acid sequence) as well as the phenotypic level (infectivity for cell lines and reactivity with *P. salmonis*-specific antiserum).

With respect to the diagnosis of Piscirickettsiosis, the following procedures have been established at AAHL:

1. Isolation and growth in fish cell cultures
2. Single-step PCR
3. Nested PCR
4. TaqMan assay
5. Immunocytochemistry
6. Immunohistochemistry

The single-step, nested and TaqMan PCR assays can also be used to detect the Tasmanian RLO. Sequence analysis of the PCR amplicons can be used to distinguish between various *P. salmonis* isolates and other RLOs, including the Tasmanian strain.

The inability of the commercial polyclonal antiserum to adequately localise the Tasmanian RLO is disappointing. The availability of such a reagent for use in immunohistochemistry would greatly assist diagnosis. An attempt was made to develop a molecular probe suitable for *in situ* hybridisation for use on tissue sections. While some progress was made such a probe has not been fully developed.

In addition, the inability of the Tasmanian RLO to grow in cell cultures has slowed, severely, progress on the characterisation of this pathogen. The RLO cannot be purified easily and thus production of antisera using conventional methods is not possible. The feasibility of using subtractive hybridisation to identify suitable gene products as antigens was investigated with little success.

Finally, those methods that are suitable for transfer to other diagnostic laboratories have been transferred to DPIWE Tasmania.

REFERENCES

Alday-Sanz V, Rodger H, Turnbull T, Adams A and Richards RH. 1994. An immunohistochemical diagnostic test for rickettsial diseases. J Fish Dis 17: 189-191.

BioManager by the Australian National Genomic Information Service (http://www.angis.org.au).

Boehringer Mannheim Application Manual. 1996. Non-radioactive *In Situ* Hybridization Application Manual, Second edition. GmbH Biochemica. Chapter 1.

Bravo S. 1994. Piscirickettsiosis in freshwater. Bull Eur Ass Fish Pathol 14: 137-138.

Bravo S and Campos M. 1989. Coho salmon syndrome in Chile. AFS/FHS Newsletter 17(3): 3.

Chen MF, Yun S, Marty GD, McDowell TS, House ML, Appersen JA, Guenther TA, Arkush KD and Hedrick RP. 2000a. A *Piscirickettsia salmonis*-like bacterium associated with mortality of white seabass *Atratoscion nobolis*. Dis Aquat Org 43: 117-126.

Chen SC, Tung MC, Chen SP, Tsai JF, Wang PC, Chen RS, Lin SC and Adams A. 1994. Systemic granulomas caused by a rickettsia-like organism in Nile tilapia, *Oreochronuis niloticus* (L.), from southern Taiwan. J Fish Dis 17: 591–599.

Chen SC, Wang PC, Tung MC, Thompson KD and Adams A. 2000b. A *Piscirickettsia salmonis*-like organism in grouper, *Epinephelus melanostigma*, in Taiwan. J Fish Dis 23: 415-418.

Corbeil S, McColl KA and Crane MStJ. 2003. Development of a TaqMan quantitative PCR assay for the identification of *Piscirickettsia salmonis*. Bull Eur Ass Fish Pathol 23: 95-101.

Crane MStJ and Williams LM. 2000. Viruses of salmonids: virus isolation in fish cell lines.
http://www.affa.gov.au/corporate_docs/publications/pdf/animalplanthealth/aquatic/virusisolation.pdf

Cvitanich JD, Garate NO and Smith CE. 1991. Isolation of a rickettsial-like organism causing disease and mortality in Chilean salmonids and its confirmation by Koch's postulate. J Fish Dis 14: 121-145.

Duguid JR and Dinauer MC. 1990. Library subtraction of *in vitro* cDNA libraries to identify differentially expressed genes in scrapie infection. Nucleic Acids Res. 18: 2789-2792.

Evelyn TPT. 1992. Salmonid rickettsial septicemia. In: Kent ML (ed) Diseases of Seawater Netpen-reared Salmonid Fishes in the Pacific Northwest. Can Spec Pub Fish Aquat Sci 116. Dept Fisheries and Oceans, Nanaimo, BC, Pp. 18-19.

Felsenstein, J. 1989. PHYLIP -- Phylogeny Inference Package (Version 3.2). Cladistics 5: 164-166.

Fryer JL, Lannan CN, Garces LH, Larenas JJ and Smith PA. 1990. Isolation of a rickettsiales-like organism from diseased coho salmon *Oncorhynchus kisutch* in Chile. Fish Pathol 25: 107-114.

Fryer JL, Lannan CN, Giovannoni SJ and Wood ND. 1992. *Piscirickettsia salmonis* gen. nov., sp. nov., the causative agent of an epizootic disease in salmonid fishes. Int J System Bacteriol 42: 120-126.

Fryer JL and Mauer MJ. 1997. The rickettsia: an emerging group of pathogens in fish. Emer Inf Dis 3: 137-144.

Gaggero A, Castro H and Sandino AM. 1995. First isolation of *Piscirickettsia salmonis* from coho salmon, *Oncorhynchus kisutch* (Walbaum), and rainbow trout, *Oncorhynchus mykiss* (Walbaum), during the freshwater stage of their life cycle. J Fish Dis 18: 277-279.

Gurtler V and Stanisich VA. 1996. New approaches to typing and identification of bacteria using the 16S-23S rDNA spacer region. Microbiol 142: 3-16.

House ML, Bartholomew JL, Winton JR and Fryer JL. 1999. Relative virulence of three isolates of *Piscirickettsia salmonis* for coho salmon *Oncorhynchus kisutch* Dis Aquat Org 35: 107-113.

Johnson D, Gautsch J, Sportsman R and Elder J. 1984. Improved technique: Utilizing nonfat dry milk for analysis of proteins and nucleic acids transferred to nitrocellulose. Gene Anal Tech 1: 3-8.

Jones SRM, Markham RJF, Groman DB and Cusack RR. 1998. Virulence and antigenic characteristics of a cultured rickettsiales-like organism isolated from farmed Atlantic salmon, *Salmo salar*, in Eastern Canada. Dis Aquat Org 33: 25-31.

Keller GH and Manak MM. 1993. DNA probes. Second Edition, Macmillan Publishers Ltd, Stockton Press, p 659.

Lannan CN, Bartholomew JL and Fryer JL. 1999. Rickettsial and Chlamydial Infections. In: Fish Diseases and Disorders (PTK Woo and DW Bruno, eds) Vol. 3, CABI International 1999, Pp.245-267.

Lannan CN and Fryer JL. 1991. Recommended methods for inspection of fish for the salmonid rickettsia. Bull Eur Ass Fish Pathol 11: 135-136.

Lannan CN, Ewing SA and Fryer JL. 1991. A fluorescent antibody test for detection of the rickettsia causing disease in Chilean salmonids. J Aquat Anim Health 3: 231-234.

Marshall S, Heath S, Henriquez V and Orrego C. 1998. Minimally invasive detection of *Piscirickettsia salmonis* in cultivated salmonids via the PCR. App Env Microbiol 64: 3066-3069.

Mauel MJ, Giovannoni SJ and Fryer JL. 1991. Phylogenetic analysis of *Piscirickettsia salmonis* by 16S, internal transcribed spacer (ITS) and 23S ribosomal DNA sequencing. Dis Aquat Org 35: 115-123.

Mauel MJ, Giovannoni SJ and Fryer JL. 1996. Development of polymerase chain reaction assays for detection, identification, and differentiation of *Piscirickettsia salmonis*. Dis Aquat Org 26: 189-195.

Mauel MJ, Giovannoni SJ and Fryer JL. 1999. Phylogenetic analysis of *Piscirickettsia salmonis* by 16S, internal transcribed spacer (ITS) and 23S ribosomal DNA sequencing. Dis Aquat Org 35: 115-123.

Office International des Epizooties. 2003. Piscirickettsiosis. Man Diag Tests Aquat Ani, Chap 2.1.13. http://www.oie.int/eng/normes/fmanual/A_summry.htm

Olsen AB, Melby HP, Speilberb L, Evensen O and Hastein T. 1997. *Piscirickettsia salmonis* infection in Atlantic salmon *Salmo salar* in Norway – epidemiological, pathological and microbiological findings. Dis Aquat Org 31: 35-48.

Reed LJ and Muench H. 1938. A simple method of estimating fifty percent endpoints. Am J Hyg 27: 493-497.

Rodger HD and Drinan EM. 1993. Observation of a rickettsia-like organism in Atlantic salmon, *Salmo salar* L., in Ireland. J Fish Dis 16: 361-369.

Wieland I, Bolger G, Asouline G and Wigler MA. 1990. Method for difference cloning: gene amplification following subtractive hybridization. Proc Nat Acad Sci USA 87: 2720-2724.

APPENDIX 1. INTELLECTUAL PROPERTY

All information arising from this project has been used for the development and/or establishment of Standard Diagnostic Procedures. No intellectual property has been identified.

APPENDIX 2: STAFF

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APPENDIX 3. STANDARD DIAGNOSTIC TECHNIQUE

Draft for endorsement by the National Aquatic Animal Health-Technical Working Group

Piscirickettsia salmonis

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SUMMARY

Piscirickettsia salmonis is the first of the previously unrecognised rickettsial pathogens of fish to be isolated, characterised, and demonstrated to be the aetiological agent of an epizootic disease. P. salmonis was first isolated in coho salmon in Chile, subsequently, piscirickettsiosis was also observed in other salmonid species (chinook salmon, Atlantic salmon, rainbow trout and masou salmon) and also in other parts of the world. It is now apparent that rickettsia-like organisms affect fish over broad host and geographic ranges. The onset of the disease most commonly occurs following transfer of fish from freshwater to seawater holding facilities. Signs of the disease include lethargy and darkening of the skin, swollen kidneys and enlarged spleen and anemia.

Identification of the agent: *Diagnosis of piscirickettsiosis is based on a range of procedures. Presumptive diagnosis is made following clinical and pathological observations. P. salmonis is confirmed following histopathological examination, isolation in tissue culture combined with identification by either immunofluorescence or immunoperoxidase staining as well as dot blot DNA hybridisation. In addition, a polymerase chain reaction technique is available for the rapid identification of P. salmonis in clinically affected animals.*

Status of Australia: *A rickettsia-like organism (RLO) was identified in Atlantic salmon reared in Tasmania, however, it has been shown that this RLO is slightly different from Piscirickettsia salmonis at the antigenic and genomic levels.*

Introduction

Piscirickettsia salmonis is an emerging, obligate, intracellular, bacterial pathogen of salmonids, first identified in Chile and later identified in Canada and several European countries (Fryer and Mauel, 1997). This Gram-negative, pleiomorphic, coccoid bacterium replicates within membrane-bound cytoplasmic vacuoles in the cells of infected fish (Fryer *et al*., 1990). Since 2001, a rickettsia-like organism (RLO) has been identified from Atlantic salmon farmed in Tasmania. However, the Tasmanian RLO differs genetically from *Piscirickettsia salmonis* isolates found overseas (in preparation).

Aetiology

Piscirickettsia salmonis epizootics in Chile have been responsible for significant economic losses to the salmonid aquaculture industry (Bravo and Campos, 1989). The Tasmanian RLO appears to be less pathogenic, causing only low-level mortality in fish (unpublished data).

Epidemiology

The onset of the disease most commonly occurs following transfer of fish from freshwater to seawater holding facilities.

Pathology

Moribund fish are described as lethargic, dark in colour. Pale gills, indicating anemia, as well as swollen kidneys and enlarged spleens are commonly observed. Large cream-to-yellow nodules may occur in the liver. Low haematocrits as well as ascites, pale liver, and petechial haemorrhages of the viscera have been observed. Additionally, there are reports of skin lesions on some affected fish (Cvitanich *et al*., 1991; Olsen *et al*., 1997).

Limitation statement

P. salmonis does not replicate on bacteriological media, but instead must be grown in cell culture, and therefore escapes detection by routine techniques used for bacterial isolation (Lannan and Fryer, 1991). In addition, *P. salmonis* will not grow if inoculated onto suitable host cells in the presence of certain antibiotics in the culture medium.

The lack of information specific to the Tasmanian RLO (e.g. susceptible cell types for *in vitro* culture, appropriate culture media, susceptibility to antibiotics, optimal growth temperature etc.) has impeded efforts to isolate it in cell culture.

Diagnostic Tests

Case definition

This standard diagnostic technique documents the methods currently used at the AAHL Fish Diseases Laboratory (AFDL) for the identification of *P. salmonis* and the Tasmanian RLO. The immunoperoxidase and PCR methods are based on those outlined in the OIE Manual of Diagnostic Tests for Aquatic Animals (OIE 2003).

Range of tests available and appropriate applications

All methods established to date have been developed for diagnostic purposes only. None of these tests have been validated for the detection of latent carriers of *P. salmonis*/RLO and therefore should NOT be used for surveillance and monitoring purposes. Thus the tests described in this SDT, identification by immunoperoxidase and PCR tests, are appropriate for diagnosis and/or *P. salmonis*/RLO exclusion in diseased fish.

Storage of samples

Samples must be maintained between 4-10^{O}C (shipping on ice packs in a styrofoam shipping container is appropriate) if isolation of the rickettsia is to be attempted. With respect to the PCR assay, the freezing of fish tissues will not affect the performance of the assay.

Tissues to be examined

Tissue samples suitable for examination include kidney, spleen, heart, brain and liver.

Tests available

For many aquatic animal diseases there is a range of tests available including histopathology, agent isolation, agent identification, electron microscopy, immuno-electron microscopy, biochemical tests, immunodiagnostic tests, molecular tests and others. This SDT documents details relevant to *P. salmonis*/RLO identification using pathogen isolation in cell culture, immunoperoxidase tests and molecular diagnostics (PCR).

Identification by Immunoperoxidase Test

Introduction

Bacteria identification by immunoperoxidase test has become a standard procedure where specific

antibodies are available. Briefly, *P. salmonis*-infected tissues are fixed and can be stored until use. The fixed preparations are incubated with a primary antibody preparation (polyclonal) which will bind to specific epitopes, if present. Excess antibody is removed by washing, and a secondary peroxidase-conjugated antibody (e.g. peroxidase anti-sheep Ig if the primary antibody was raised in sheep) is added. After an incubation period, excess conjugate is removed by washing and peroxidase substrate (e.g. AEC) is added and colour is allowed to develop. Finally, following washing in water, cells are counterstained with Mayer's haematoxylin, rinsed in water and blued with Scott's tap water. Thus, if any bacteria recognised by the primary antibody are present, a positive colour reaction will occur.

List of equipment

-Qualtex incubator
-Refrigerator
-Microscope slides (frosted ends)
-Coverslips
-Immunostaining chambers
-Inverted light microscope fitted with 4X and 10X objectives
-Pangalark slide storage system

Reagents

-Phosphate Buffered Saline (pH 8.0)
-80% (v/v) acetone in water
-0.05% (v/v) Tween 20 (BDH product # 66368) in PBSA
-1% (w/v) skim milk powder (Carnation or Diploma - Bonlac Foods Ltd) solution in PBSA
-Anti-*P. salmonis* (LF-89 isolate) sheep polyclonal antibodies (Microtek International cat # SPS01)
-Peroxidase labelled donkey anti-sheep IgG (Sigma cat # A-3415)
-3-amino-9-ethyl carboxyzole (AEC, Sigma cat # A-5754)
-Dimethylformamide (BDH Product # 10322)
-Acetate buffer 0.05M (pH 5.0) (BDH Analar Product # 10236)
-Hydrogen peroxide (BDH Analar Product # 10366)
-Deionised water
-Mayer's haematoxylin (Lillie's Modification; Dako Code S3309)
-Scott's tap water
-AquaPerm Mounting Medium (IMMUNON™ cat # 484985)

Quality control

For immunoperoxidase testing, separate control tissues are set up in parallel with the tissues of the test samples. Positive controls are tissues from fish infected with a known *P. salmonis* LF-89 isolate. These and a known uninfected tissue (negative control) are processed on the same day as the test samples. Antibody preparations for diagnostic tests are stored at -20^{O}C until use and check-tested on a regular basis.

The working dilutions of each diagnostic reagent (antibodies, conjugates, substrates) need to be determined prior to use and check-tested on a regular basis.

Procedures

Immunoperoxidase test procedure

Prepare 1% (w/v) and 0.1% (w/v) skim milk powder solution in PBSA for antibody dilution

Dilute the anti-*P. salmonis* primary sheep polyclonal antibody (1/100) and normal sheep serum (1/100) in 1% skim milk powder solution in sterile PBSA. After washing in PBSA, the sections are incubated for 1 h at 37^{o}C with a peroxidase labelled donkey anti-sheep IgG diluted 1:200 in 0.1% skim milk in PBSA. Following a further wash in PBSA, the sections are incubated with freshly prepared substrate solution (2mg AEC, 200µL dimethylformamide, 10mL 0.05 M acetate buffer pH 5.0, 5µL 30% (v/v) hydrogen peroxide for 20 min. at room temperature. The sections are rinsed in tap water, counterstained with Mayer's haematoxylin and mounted in GelTol (Immunon) for light microscopic examination.

Interpretation

Positive reaction: Grainy, focal, brick-red staining of cells indicates presence of *P. salmonis* LF-89 isolate (figure 1a) and Tasmanian RLO (figure 1b) identified by the diagnostic polyclonal antiserum.

Negative reaction: No red staining apparent (figure 1c).

Background staining: Non-grainy, non-focal, pale, pinkish staining may occur and could be due to any of a number of reasons.

Identification by PCR

Introduction

Polymerase chain reaction (PCR) has become an important diagnostic tool both in human and veterinary medicine. As with many infectious diseases, especially where the aetiological agent

cannot be easily propagated *in vitro*, development of procedures for the specific and sensitive detection and identification of the agent by PCR has provided some diagnostic capability. Even where the causative agent can be propagated, if specific PCR primers are available, PCR provides a powerful tool for the rapid identification of the agent. Furthermore, subsequent sequencing of any PCR product may allow a molecular epidemiological analysis of the agent, and, for exotic diseases in particular, this may be very important in control, prevention and eradication.

However, there are some pitfalls that need to be noted. Firstly, since only a small amount of tissue is used for DNA extraction, it is therefore possible to harvest part of the tissue that does not contain any micro-organism, yielding a false negative diagnosis. Secondly, for nested PCRs especially, cross-contamination between reaction tubes can occur, potentially yielding a false-positive diagnosis. While these pitfalls continue to exist, reliance on PCR results alone for a diagnosis is not recommended and it is important that other, additional tests (e.g. agent isolation, histology and diagnostic immunoassays) are used, if available. In this respect, immunoassays using pan-specific antibodies are very powerful for detection and identification of all variants of an aetiological agent while PCR technology has the potential to provide capability for distinguishing variants.

Thus the use of PCR as a diagnostic tool requires a large investment in human and physical resources. Specialised equipment for exclusive molecular diagnosis by qualified and experienced operators is essential. All equipment and procedures need to be strictly controlled to ensure validity of results. It is recommended that diagnostic PCR activities be carried out in a dedicated suite of laboratories with strict control on the transfer of materials and personnel in and out of the suite. Diagnostic laboratories are likely to have designed their PCR facilities and generic procedures according to their own specific requirements and therefore only general direction can be given here.

Reagents

Reagents stored at –20℃
-Taq polymerase (Promega cat # M1861)
-dNTPs (1.25mM) (Promega cat # 12957510, 12957611, 13466203, 13466310)
-Mg free buffer 10X (Promega cat # 15913012)
-$MgCl_2$ (25mM) (Promega cat # 15246902)
-100% Ethanol AR grade
-70% Ethanol
-Primers (18µM)
-HotstarTaq Mastermix (QIAGEN Cat # 203443)
-100bp DNA ladder & loading dye (Promega cat # G2101)

Reagents stored at room temperature
QIAamp DNA Mini Kit (QIAGEN Cat # 51304):
-DNA columns
-Buffer ATL
-Buffer AL
-Buffer AW1
-Buffer AW2
-Buffer AE
-Proteinase K
-Agarose (BIORAD cat#162-0134)
-Ethidium bromide (BIORAD cat #161-0430)
-40 x TAE Buffer (Promega cat # 428A)

List of equipment

Apart from the normal range of equipment required in the standard diagnostic laboratory (e.g. refrigerators, freezers, vortex mixers, micropipettes, biological safety cabinets, centrifuges, balances, microwave oven, thermometers), specialised equipment required to undertake diagnostic PCR may include dry heat blocks, thermocycler, gel electrophoresis equipment, UV transilluminator, camera system and nucleic acid sequencer.

Quality control

Molecular diagnosis should be operated under an ISO 17025 accredited and audited quality assurance program. Thus, such a program would include initial evaluation of kits and validation of performance; ongoing internal evaluation through mandatory use of appropriate quality control samples where available; and performance monitoring through quality assessment or proficiency programs.

External quality control samples over the appropriate range of testing must be obtained or manufactured wherever possible. Wherever possible, quality control samples should be included in every assay run and the data presented so that run-to-run performance can be monitored. Positive, negative and reagent controls should be conducted as specified in the protocol. As a norm, formalin fixed controls would be conducted with formalin fixed test samples and appropriate unfixed controls would be conducted with fresh tissue, culture supernatants or other test samples. Stocks of controls should be established. These controls should be evaluated prior to storage and used in a check-testing regimen and as controls for the conduct of disease investigations.

Procedures

Sample preparation

Due to the sensitivity of PCR tests, care at every step of sample preparation must be taken to ensure that cross-contamination of diagnostic samples does not occur. Thus all instruments and sample containers must be clean and uncontaminated i.e. not pre-exposed to aquatic pathogens. Wherever possible, it is recommended that disposable containers are used.

At AAHL, samples would be handled and processed using sterile disposable single-use containers, instruments and reagents to minimise the risks of contamination of the samples. As a general principle, samples to be used in the PCR suite for molecular diagnosis will be inactivated by an approved method prior to movement to the PCR suite.

Inactivation will be carried out by the following procedures by staff approved to work with the categories of agents.

For each fish sample, approximately 20mg of tissues are harvested using sterile scissors and tweezers and put in a sterile 1.5mL vial (conical bottom). Tissues are mashed using a disposable plastic green pestle and each sample receives 180µL of ATL buffer. All labelled vials are then put in a jar.

Nucleic acids are extracted from submitted samples in the Biological Safety Cabinet Class II in the PCR suite.

Nucleic acid extraction

Nucleic acid (including *P. salmonis* DNA) is obtained from cell culture supernatant or tissue samples using the QIAamp DNA extraction kit following manufacturer's instructions.

P. salmonis-specific PCR (Mauel *et al*., 1996).

Primary amplification

A) The PCR mixture for a single sample consists of the following reagents: 9.5µL of deionised sterile water; 12.5µL of HotStar Taq Master mix, 0.5µL of the universal nested (Eubacterial 16S rDNA) forward primer Eub-B AGAGTTTGATCMTGGCTCAG (18µM); 0.5µL of the reverse primer Eub-A: AAGGAGGTGATCCANCCRCA (18µM); and 2µL of extracted DNA. For multiple samples, the volumes are multiplied appropriately. The mixture is incubated in an automatic thermal cycler (Perkin Elmer GeneAmp 2400) that is programmed for: one cycle at 94°C for 15 minutes (activation of the Hotstar Taq polymerase); 35 cycles at 94°C for 1 minute (denaturation), 50°C for 2 minutes (annealing) and 72°C for 3 minutes (extension); and, finally, one cycle at 72°C for 7 minutes (final extension).

B) Alternatively, the following reagents can be used (per sample) for the primary amplification: 12.5µL of deionised sterile water, 4µL dNTPs, 2.5µL $MgCl_2$ free buffer10X, 1.5µL $MgCl_2$, 1µL Eub-B primer (18µM), 1µL Eub-A primer (18µM), 0.5µL Taq polymerase, 2µL of sample DNA. The mixture is incubated in an automatic thermal cycler (Perkin Elmer GeneAmp 2400) that is programmed for: one cycle at 94°C for 2 minutes; 35 cycles at 94°C for 1 minute, 50°C for 2 minutes and 72°C for 3 minutes; and, finally, one cycle at 72°C for 7 minutes.

Nested PCR amplification

The second amplification is performed by adding 2µL of the first PCR products to the reaction mixtures described above (methods A or B) except for the presence of the *P. salmonis* specific primers PS2S (forward, CTAGGAGATGAGCCCGCTTG) and PS2AS (reverse, GCTACACCTGCGAAACCACTT) instead of Eub-A and Eub-B, under the following reaction conditions: 1 cycle at 94°C for 15 minutes (if using Hotstar Taq, otherwise 1 cycle at 94°C for 2 minutes for normal Taq); 35 cycles at 94°C for 1 minute, 61°C for 2 minutes and 72°C for 3 minutes; and, finally, one cycle at 72°C for 7 minutes.

Amplified DNA (size: 476 bp) is detected by agarose gel electrophoresis (Figure 2).

Interpretation

At the completion of the PCR, specific amplicons of the correct size are identified by agarose gel electrophoresis:

The negative control sample must have no evidence of specific amplicon.

A positive control sample must yield a specific *P. salmonis* amplicon of 476 bp.

Amplicons of the correct size are then eluted from the gel, and the DNA sequence is determined (by using the PCR primers PS2S and PS2AS as sequencing primers).

Sequence identity and genotype are determined by a Blast search of the Genbank database.

An assay is valid only when all controls yield the expected results.

Isolation in cell culture

Five standard fish cell lines, CHSE-214, FHM, EPC, RTG-2, BF-2, were tested for their susceptibility to infection by the three reference strains of *P. salmonis* (c.f. OIE Aquatic Manual for detailed method). All five cell lines were shown to be susceptible (e.g. Figure 3) with CHSE-214 being most susceptible followed by BF-2. However, none of the cell lines appear to be susceptible to the Tasmanian RLO which has yet to be isolated in cell culture. It appears that the RLO demonstrates different culture requirements yet to be identified.

Acknowledgements

Bacterial DNA from *P. salmonis* isolates LF-89, ATL-4-91 and NOR-92 were obtained from Dr John L. Fryer (Oregon State University, USA). Paraffin blocks of *P. salmonis* (LF-89) infected fish tissues were obtained from Dr Marcia House and Dr Jim Winton (Western Fisheries Research Center, Seattle, USA.)

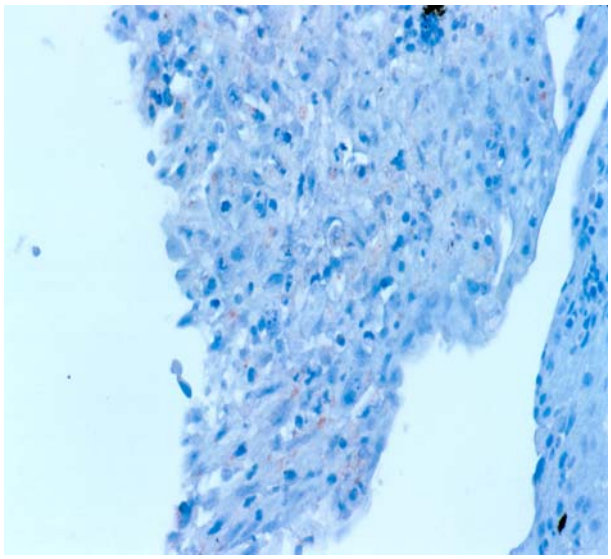

Figure 1b. Immunohistochemistry of Tasmanian RLO infected fish tissue, using sheep anti-LF-89 *P. salmonis* polyclonal antibodies. Magnification 250X. Note that the staining is weaker due to antigenic determinant differences between *P. salmonis* and the RLO.

Figures

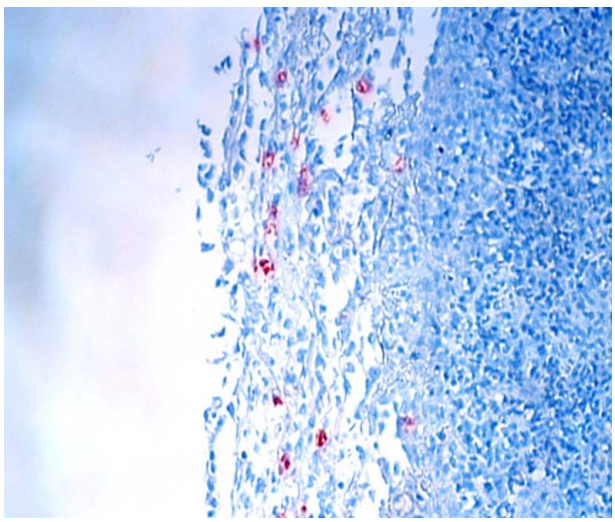

Figure 1a. Immunohistochemistry of *P. salmonis* (LF-89 isolate) infected fish tissue, using sheep anti-LF-89 *P. salmonis* polyclonal antibodies. Magnification 250X.

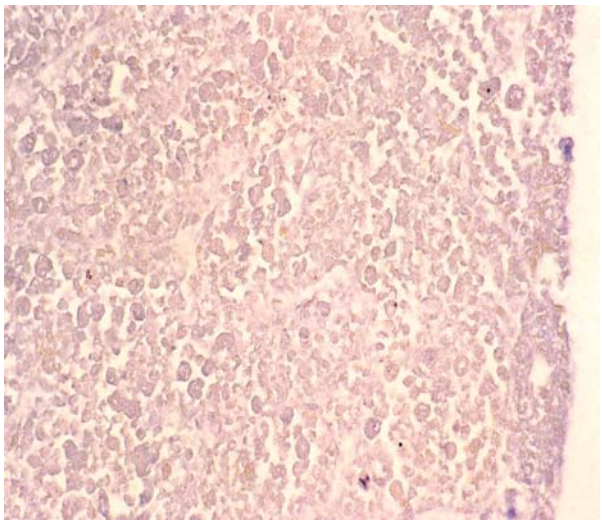

Figure 1c. Immunohistochemistry of Tasmanian RLO infected fish tissue, using non-immune sheep polyclonal antibodies. Magnification 400X.

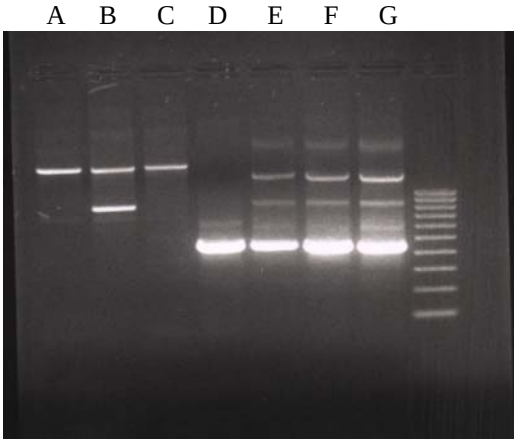

Figure 2. The *P. salmonis*-specific nested PCR. Amplicons (bright bands) of 487 base pairs in size are seen in lanes D, E, F and G. Wells E, F, and G contained DNA samples extracted from supernatants of cell cultures infected with the exotic *P. salmonis* isolates LF-89, ATL-4-91, and NOR-92, respectively. Lane D contained a DNA sample extracted from fish tissue infected with the Tasmanian Rickettsia-like organism (RLO). Lanes A, B, and C contained DNA samples from unrelated fish bacteria used as negative controls. The last lane on the right-hand-side of the gel contained the 100 bp ladder. Note that in addition to the specific bands in lanes D, E, F and G, there are some non-specific bands in each lane, including lanes D, E, F and G.

Figure 3. Growth of *P. salmonis* in cell culture. Photomicrograph of CPE, typical of *P. salmonis*, developing in cell cultures of CHSE-214 cell line. Magnification 40X, CHSE-214 cultures at day 11 post-infection with *P. salmonis* NOR-92 strain (Norway) in the presence of 100µg/mL ampicillin. 100µL of a 10^4 $TCID_{50}$/mL stock suspension of *P. salmonis* was used to infect cells.

References

Bravo S and Campos M (1989). Coho salmon syndrome in Chile. AFS/FHS Newsletter 17(3): 3.

Cvitanich JD, Garate NO, Smith CE (1991). Isolation of a rickettsial-like organism causing diseases and mortality in Chilean salmonids and its confirmation by Koch's postulate. J Fish Dis 14: 121-145.

Fryer JL and Mauel MJ (1997). The rickettsia: an emerging group of pathogens in fish. Emer Infect Dis 3: 137-144.

Fryer JL, Lannan CN, Garces LH, Larenas JJ, Smith PA (1990). Isolation of a rickettsiales-like organism from diseased coho salmon *Oncorhynchus kisutch* in Chile. Fish Pathol 25(2): 107-114.

Lannan CN and Fryer JL (1991). Recommended methods for inspection of fish for salmonid rickettsia. Bull Eur Assoc Fish Pathol 11: 135-136.

Mauel MJ, Giovannoni SJ, Fryer JL (1996). Development of polymerase chain reaction assays for detection, identification, and differentiation of *Piscirickettsia salmonis*. Dis Aquat Org 26: 189-195.

OIE (2003). Piscirickettsiosis. In: Manual of Diagnostic Tests for Aquatic Animals. Chapter 2.1.1.8. Office International des Epizooties 4th Edition, 2003), Paris.

Olsen AB, Melby HP, Speilberb L, Evensen O, Hastein T (1997). *Piscirickettsia salmonis* infection in Atlantic salmon *Salmo salar* in Norway – epidemiological, pathological and microbiological findings. Dis Aquat Org 31(1): 35-48.