Australian and New Zealand Standard Diagnostic Procedures (ANZSDP) for *Piscirickettsia salmonis* (piscirickettsiosis) and piscine rickettsia-like organisms

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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. *Piscirickettsia salmonis* was first isolated in coho salmon in Chile, and the bacterium has subsequently been observed in other salmonid species (Chinook salmon, Atlantic salmon, rainbow trout and masu salmon) across many parts of the world. It is now known that rickettsia-like organisms affect fish over broad host and geographic ranges. In Chile 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 anaemia.

Identification of the agent

Diagnosis of piscirickettsiosis is based on a range of procedures. Presumptive diagnosis is made following clinical and pathological observations. *Piscirickettsia salmonis* is confirmed following histopathological examination, isolation in tissue culture on solid medium or in liquid medium, combined with identification by either immunofluorescence or immunoperoxidase staining. In addition, polymerase chain reaction (PCR) techniques are available for the rapid identification of *P. salmonis* in clinically affected animals.

Status of Australia and New Zealand

Piscirickettsia salmonis is exotic to Australia and New Zealand. In 2001 a rickettsia-like organism (RLO) was identified in Atlantic salmon farmed in Tasmania. However, the Tasmanian RLO differs at the genetic and antigenic level from *P. salmonis* isolates found overseas (Corbeil et al., 2005). In 2015, RLO were identified in farmed Chinook salmon in New Zealand (Brosnahan et al., 2017).

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1. Diagnostic overview

Introduction

Piscirickettsia salmonis is a facultative 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, pleomorphic, coccoid bacterium replicates within membrane-bound cytoplasmic vacuoles in the cells of infected fish (Fryer et al., 1990). Molecular phylogenetic analysis based on sequencing of the 16S rRNA gene placed *P. salmonis* in a new family of Piscirickettsiaceae within the class of γ-proteobacteria.

Aetiology

Piscirickettsia salmonis epizootics in Chile have been responsible for significant economic losses to the salmonid aquaculture industry; mortality rates were reported to be 30% to 90% among coho salmon (Bravo and Campos, 1989). *Piscirickettsia salmonis*-like organisms have recently been isolated from non-salmonid fish in various countries Chen et al., 1994; Chen et al, 2000, Chen et al., 2000a, Arkush et al., 2005, McCarthy et al., 2005, Thomas and Faisal 2009). The relationships of most of these organisms to *P. salmonis* have not been fully elucidated, but the organism isolated from white seabass (*Atractoscion nobilis*) is genetically and serologically indistinguishable from *P. salmonis* (Chen et al., 200a). The complete genomic sequence of *P. salmonis* (LF-89T strain) was obtained in 2001 (Valenzuela et al., 2001). Complete genomic sequences from more strains of *P. salmonis* as well as rickettsia-like organisms (RLOs) would allow the refinement of their phylogeny.

Clinical signs

A range of gross signs of disease may be present in salmonids infected with *P. salmonis*. Severely affected fish are dark, anorexic and lethargic. They often swim near the water surface or at the edges of the cages. Fish with milder infections may appear normal. Infections of the brain may cause erratic swimming behaviour (Skarmeta et al., 2000).

Skin lesions, appearing as small white patches that can progress to shallow ulcers, may also be present on some fish. The most consistent external sign seen during *P. salmonis* infections is pale gills indicating anaemia but this is not pathognomonic for the disease.

In common with many systemic, chronic inflammatory diseases of salmonids, the internal signs include a swollen and discoloured kidney and an enlarged spleen. Ascites and haemorrhages on the visceral fat, stomach, swim bladder and body musculature can also occur (Cvitanich et al., 1991; Schäfer et al., 1990). Hallmark internal lesions of the disease are found in the liver, which may exhibit large, whitish or yellow, multifocal, coalescing, pyogranulomatous nodules. These lesions often rupture, resulting in shallow crater-like cavities in the liver.

Epidemiology

Transmission of *P. salmonis* to coho salmon can occur via skin, gills and intestine (Smith et al., 2004). The disease has been primarily reported in marine fish farms, and has also been observed in freshwater facilities (Bravo, 1994; Gaggero et al., 1995). Horizontal transmission occurs in saltwater and freshwater (Cvitanich et al., 1991; Almendras et al., 1997). It is not known whether transmission can occur via vectors., However, vertical transmission under experimental conditions has been demonstrated (Larenas et al., 2003). The onset of the disease most commonly occurs following

transfer of fish from freshwater to seawater holding facilities. Fish of all ages, from smolts to market size (Cvitanich et al., 1991), are susceptible to the disease. The disease begins approximately 1 month after fish are introduced into the seawater net pens.

Although useful, antibiotics have limited effects on the disease (Cabello, 2006). Management of the disease is based on a range of husbandry practices, including the application of immunostimulants of unproven efficacy and the control of vertical transmission by an expensive selection procedure during reproduction (Wilhelm et al., 2006). Periods of fallowing have been one approach to limit the spread of the pathogen (Olivares and Marshall, 2010). Although experimental vaccines showed some efficacy in laboratory conditions, commercial vaccines have not yielded significant protection against piscirickettsiosis in the field (Smith et al., 1997; Kuzyk et al. 2001; Leal and Woywood, 2007; Marshall et al., 2007; Tobar et al., 2011; Rozas and Enriquez, 2014).

Occurrrence and distribution

In Tasmania, three RLO serotypes/serovars have been identified. They originate from the Macquarie Harbour, Tamar River and South East Atlantic salmon marine production zones. While RLO isolates have been obtained from all three major marine production areas, there is only evidence of clinical cases of RLO-associated disease in the south east production zone (DPIPWE unpublished information).

In New Zealand, three RLO strains have identified. Genetic testing showed that the RLO are different to the *P. salmonis* found in Chilean salmon farms and one strain (NZ-RLO1) has been shown to be similar to the Tasmanian RLO over two genes (internal transcribed spacer region and 16S rRNA) (Brosnahan et al., 2017). Historical tissue samples from a 2012 disease outbreak have been tested using newer diagnostic techniques. This has shown the rickettsia-like organism was present then and is likely to have been in New Zealand for some years and may be widespread in marine environment (Ministry for Primary Industries—Manatū Ahu Matua).

Pathology

The most marked histological changes in infected fish are found in the liver, kidney, spleen and intestine with lesser changes in the brain, heart, ovary and gills (Schäfer et al., 1990; Branson and Diaz-Munoz, 1991; Cvitanch et al., 1991; Olsen et al., 1997; Palmer et al., 1997). Multifocal necrosis of hepatocytes, accompanied by a chronic inflammatory infiltrate of mononuclear cells, is seen in the liver. Vascular and perivascular necrosis are also evident in the liver, and intravascular coagulation resulting in fibrin thrombi within major vessels is a common finding. The focal areas of necrosis underlie the pale circular lesions seen grossly in more chronically infected fish. In more acute infections, the coalescence of necrotic areas results in a more mottled appearance of the liver rather than discrete nodules.

Granulomatous inflammation also occurs in the interstitium of the kidney and parenchyma of the spleen. Vascular changes similar to those in the liver may also be seen in the kidney and spleen. Meningitis, endocarditis, peritonitis, pancreatitis, and branchitis may be seen with accompanying chronic inflammatory and vascular changes similar to those in the liver and haematopoietic organs.

Using high magnification, examination of lesions reveals aggregates of the organism in the cytoplasm of degenerated hepatocytes and in macrophages. Infected macrophages are usually hypertrophied and replete with cellular debris. In tissue sections stained with haematoxylin and eosin (H&E) the organisms appear as basophilic or amphophilic spheres, about 1 μm in diameter. In recent years, the gross pathology of piscirickettsiosis has evolved towards the presentation of multiple diffuse skin

ulcers all over the body. This pathology may also include cavernous deficits in the skeletal muscle (Rozas and Enriquez, 2014).

P. salmonis versus Tasmanian rickettsia-like organism

Section written by Graeme Knowles, Veterinary pathologist, Animal Health Laboratory, DPIPWE Tasmania.

P. salmonis infection in Atlantic salmon causes some similar gross findings, but also notable differences, to Tasmanian rickettsia-like organism (RLO) infection in Tasmanian farmed Atlantic salmon, which is characterized by high morbidity rates and reduced feed intake but relatively low mortality rates (less than 10% of affected stock). The pale gills and whitish to yellow multifocal to coalescing nodules in the liver, characteristic of *P. salmonis* infection (Birrel et al., 2003; Freyer and Hendrick, 2003; Rozas and Enriquez, 2014) along with swollen grey kidneys are absent in Tasmanian RLO infected Atlantic salmon. Similar to *P. salmonis* infection, Tasmanian RLO will cause petechia of visceral fat and multifocal pallor of the liver (Ferguson, 2006). This pallor, in Tasmanian RLO, is due primarily to hepatocyte necrosis (Morrison et al., 2016) whereas in *P. salmonis* infection pyogranulomatous inflammation as well as necrosis contributes to the pallor. Gross findings seen only in Tasmanian RLO infected Atlantic salmon include congestion of posterior intestinal (hindgut) serosa, red (hyperaemic) anus and haemorrhage in the anterior chamber of the eye (blood-spot) (Morrison et al., 2016).

There are key differences between histopathological findings in *P. salmonis* and Tasmanian RLO infected Atlantic salmon. *P. salmonis* infection causes replacement of hepatic tissue or renal haematopoietic tissue by pyogranulomatous inflammation, inflammation of the lamina propria in the intestines and branchitis (Birrel et al., 2003; Ferguson, 2006).

These findings are not seen in Tasmanian RLO infected fish (Ferguson, 2006). On the other hand necrotizing hepatitis and meningitis are seen in both *P. salmonis* and Tasmanian RLO infected Atlantic salmon (Ferguson, 2006). In Tasmanian RLO infection the necrotizing hepatitis is often accompanied by mild non-supportive inflammation and encephalitis will occur alongside meningitis (DPIPWE unpublished data). Additional histopathological findings in Tasmanian RLO infected Atlantic salmon include steatitis of visceral fat, epicarditis, myocarditis and posterior uveitis of anterior chamber of eye (Ferguson, 2006). In Tasmanian RLO infected Atlantic salmon, 1 µm diameter coccoid amphophilic intrahistiocytic RLOs are sometimes seen with the multi-organ inflammation on haematoxylin and eosin staining (Morrison et al., 2016). These organisms can be highlighted by Giemsa stain (Ferguson, 2006).

In summary, characteristic gross changes of *P. salmonis* infection such as pale gills, pale white to yellow hepatic foci or swollen grey kidneys are not seen in Tasmanian RLO infected Atlantic salmon. However, haemorrhage (including petechia) of visceral fat and multiple pale foci in liver can be caused by both pathogens. Microscopic changes characteristic of *P. salmonis* infection, including pyogranulomatous hepatitis, or necrotizing hepatitis, pyogranulomatous nephritis (with loss of haematopoietic tissue) and branchitis are not seen in Tasmanian RLO infected Atlantic salmon. Both Tasmanian RLO and *P. salmonis* infected fish can develop necrotizing hepatitis and meningitis.

Diagnostic tests

Piscirickettsia salmonis can be detected using several methods/techniques including; smears or impressions of tissues or infected cell cultures on glass, fixed and stained with Gram, Giemsa, acridine orange or methyl blue stain for direct observation of the bacterium within host cells (Fryer et al., 1990; Lannan and Fryer 1991) *Piscirickettsia salmonis* can be grown in cultured cells and in bacteriological media (Mauel et al., 2008; Mikalsen et al., 2008; Gomez et al 2009; Yanez et al., 2012;

Yanez et al., 2013; Yanez et al., 2014). Following initial detection, the identity of *P. salmonis* must be confirmed by either serological methods such as immunofluorescence (IFAT) (Lannan et al., 1991; Larenas et al., 2003), an enzyme-linked immunosorbent assay (ELISA) (Aguayo et al., 2002), immunohisto-chemistry (Alday-Sanz et al., 1994) or a recently developed single-dilution filtration-assisted chemi-luminometric immunoassay (SD FAL-ELISA) (Wilda et al., 2012), or molecular techniques such as PCR (Mauel et al., 1996; Marshall et al., 1998; Corbeil et al., 2003; Karatas et al., 2008; Tapia-Cammas et al., 2011) or in situ hybridization assay (Venegas et al., 2004). Not all these methods are described in this document.

Case definition

This standard diagnostic procedure documents the methods for the identification of *P. salmonis* following submission of samples from fish presenting clinical signs. The histology, immunohistochemistry, and conventional PCR methods are based on those first outlined in the OIE Manual of Diagnostic tests for Aquatic Animals (OIE, 2016).

Range of tests available and appropriate applications

All methods established to date have been developed for detection and identification of *P. salmonis* and the Tasmanian RLOs in diseased fish. Some of these tests (PCR and culture) are known to detect and identify NZ-RLOs. None of these tests have been validated for the detection of sub-clinical carriers of these pathogens and therefore should **not** be used for surveillance and monitoring purposes. The tests described in this ANZSDP, for identification by immunohistochemical and PCR tests, are appropriate for diagnosis and/or exclusion of *P. salmonis* and the Tasmanian RLOs in diseased fish.

Storage of samples

Samples must be maintained between 4°C and 10°C and processed as soon as practicable after sampling (shipping on ice packs in a styrofoam shipping container is appropriate) if isolation of the bacterium is to be attempted. Freezing fish tissues will not affect the performance of PCR assays.

Tissues to be examined

Tissues suitable for examination include kidney and liver.

Tests available

This ANZSDP describes methods for the identification of *P. salmonis* and RLO-like organisms that have previously been detected in Tasmania and New Zealand using histology, immunohistochemical tests, molecular diagnostics (PCR and subsequent sequencing) and pathogen isolation in cell culture and solid agar medium.

Note: These immunohistochemical tests have not been validated for use on NZ-RLO.

2. Test methods

Histology

1) Preserve visceral organs in formalin-based fixative and process for routine histology.
2) Stain histological slides with H&E or Giemsa.
3) Examine macrophages within the kidney interstitium, spleen or blood, or hepatocytes within liver lesions, for the presence of multiple, spherical, basophilic or amphophilic bodies (by H&E) or dark blue bodies (by Giemsa), approximately 0.5 µm to 1.5 µm diameter in the cytoplasm.

Giemsa stain

1) Preparation of tissue culture supernatant, smears or impressions of the kidney, liver, and spleen are prepared, air dried, and fixed for 5 minutes in absolute methanol.
2) Immerse slides in a working solution of Giemsa stain for 30 minutes.
    a) Stock solution: 0.4 (w/v) Giemsa powder in buffered methanol solution, pH 6.9 (commercially available).
    b) Working solution: Stock solution diluted 1/10 in phosphate buffer pH 6.0 (0.074 M NaH2PO4, 0.0009 M Na2HPO4).
3) Destain with tap water.
4) Examine slides under oil immersion. Tissue smears from infected organs show darkly stained pleomorphic organisms occurring in coccoid or ring forms, frequently in pairs, with a diameter of 0.5 µm to 1.5 µm.

Identification by immunohistochemical (immunoperoxidase) test

With the contribution of Jenni Harper, CSIRO, AAHL, Victoria.

Introduction

Bacterial 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 (monoclonal) that will bind to specific epitopes, if present. Excess antibody is removed by washing, and a secondary peroxidase-conjugated antibody (for example, peroxidase anti-sheep Ig if the primary antibody was raised in sheep) is added. After an incubation period, excess conjugate is removed by washing, peroxidase substrate 3-amino-9-ethyl carboxyzole (AEC) is added and color is allowed to develop. Finally, following rinsing in water, cells are counterstained with Mayer's haematoxylin, rinsed in water and developed with Scott's solution. Thus, if any bacteria recognized by the primary antibody are present, a positive color reaction will occur.

List of equipment

- Incubator 37°C
- Refrigerator
- Microscope coated slides (SuperFrost™)
- Coverslips

- Immunostaining chambers
- Light microscope fitted with 4× and 10× objectives

Reagents

- TRIS-HCl 0.005 M buffer pH 7.6 (Table 1)
- 80% (v/v) acetone in water
- Tween 20 0.05% (v/v) in 0.005 M TRIS-HCl buffer pH 7.6
- Phosphate buffered saline A pH 7.4
- Skim milk powder solution 1% (w/v) in 0.005 M TRIS-HCl buffer pH 7.6
- Anti-*P. salmonis* IgG monoclonal antibody (clone 7G4/D9) (Ango, catalogue number FM-140AX-5).
- Anti-mouse Ig (Dako Envision Flex/HRP K8010)
- 3-amino-9-ethyl carboxyzole
- Dimethylformamide
- Acetate buffer 0.05 M (pH 5.0) (Table 1)
- Hydrogen peroxide 30% (v/v)
- Deionised water
- Mayer's haematoxylin
- Scott's solution (Table 1)
- Mounting medium

The kit K8002 Dako EnVision™ Flex/HRP +, Mouse, High pH, provides all reagents required for the immunoperoxydase test (*P. salmonis* primary antibody excepted).

Table 1 Base ingredients for immunohistochemical (immunoperoxidase) test reagents

Reagent	**Amount**
TRIS-HCl 0.005 M buffer pH 7.6	
NaCl	8.1 g
TRIS (TRIS hydroxymethylmethylamine)	0.6 g
Distilled water	3.8 mL
NaCl	to 1.0 L
Acetate buffer 0.05 M (pH 5.0)	
Sodium acetate $Na_2C_2H_3O_2.3H_2O$	6.8 g
Distilled water	1.0 L
Use glacial acetic acid (approx. 1.0 mL) to alter pH to 5.0	---
Scott's solution	

Reagent	Amount
$NaHCO_3$	7.0 g
$MgSO_4$	40.0 g
Distilled water	2.0 L

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* isolate (for example: LF-89T). These tissues are no longer available in Australia and would need to be produced by experimental infection at the Australian Animal Health Laboratory (AAHL) when funding is made available. Positive control tissues 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°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.

Immunoperoxidase test procedure

1) Dewax slides through three baths of absolute ethanol (2 minutes in each bath), once in 70% ethanol for two minutes, rinse to running tap water. Leave in distilled water if delayed before retrieving slides.
2) Add H_2O_2 (10%) 10 minutes on each section (antigen retrieval procedure).
3) Rinse with Tris-HCl buffer.
4) Add Proteinase K (5 ug/mL) for 5 minutes.
5) Rinse with Tris-HCl buffer.
6) Prepare 1% (w/v) and 0.1% (w/v) skim milk powder solution in phosphate buffered saline A (PBSA) for antibody dilution or use the Dako EnVision diluent from kit.
7) Dilute the anti-*P. salmonis* primary mouse monoclonal antibodies (1/10) and non-specific mouse monoclonal (1/10) in 1% skim milk powder solution in sterile PBSA or use the Dako EnVision diluent from the kit.
8) Add the primary antibody solution at room temperature (20°C) for 45 minutes.
9) Rinse sections with Tris-HCl buffer.
10) Incubate with a secondary antibody peroxidase labelled anti-mouse Ig, undiluted, at room temperature (20°C) for 20 minutes.
11) Rinse in Tris-HCl buffer.
12) Incubate with freshly prepared substrate solution (2 mg AEC, 200 µL dimethylformamide, 10 mL 0.05 M acetate buffer pH 5.0, 5 µL 30% (v/v) hydrogen peroxide) at room temperature for 20 minutes or for 5 minutes if using DAB reaction Dako EnVision kit.
13) Rinse the sections in tap water.
14) Counterstain with Mayer's haematoxylin.
15) Rinse in Scott's solution and mount for light microscopic examination.

Interpretation

Positive reaction

Grainy, focal, brick-red staining of cells indicates presence of Tasmanian RLO and P. salmonis (Figure 1, Figure 2) identified by the diagnostic IgG monoclonal antibody.

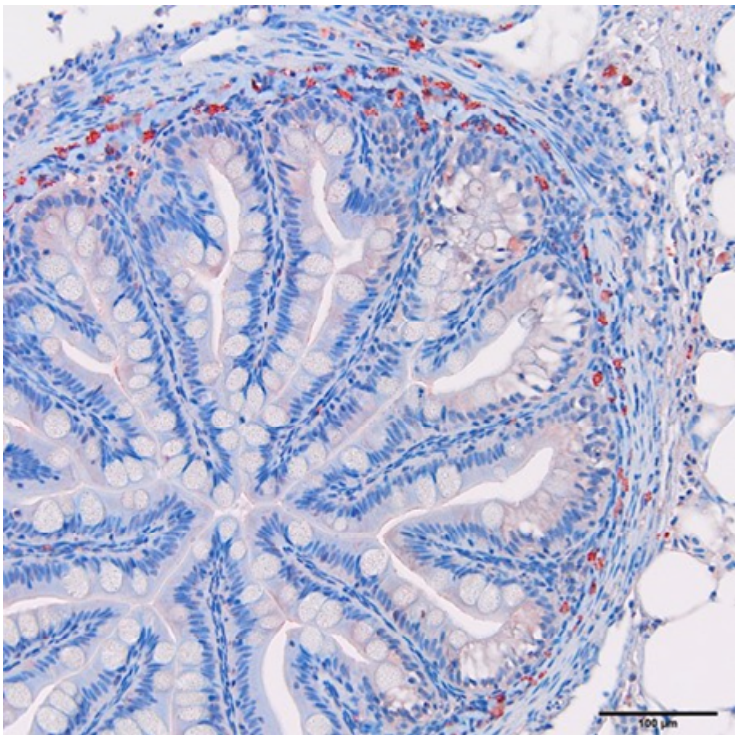
Negative reaction

The non-specific monoclonal antibody (anti-WSSV) produces only a faint background on the infected tissues (Figure 3, Figure 4).

Background staining

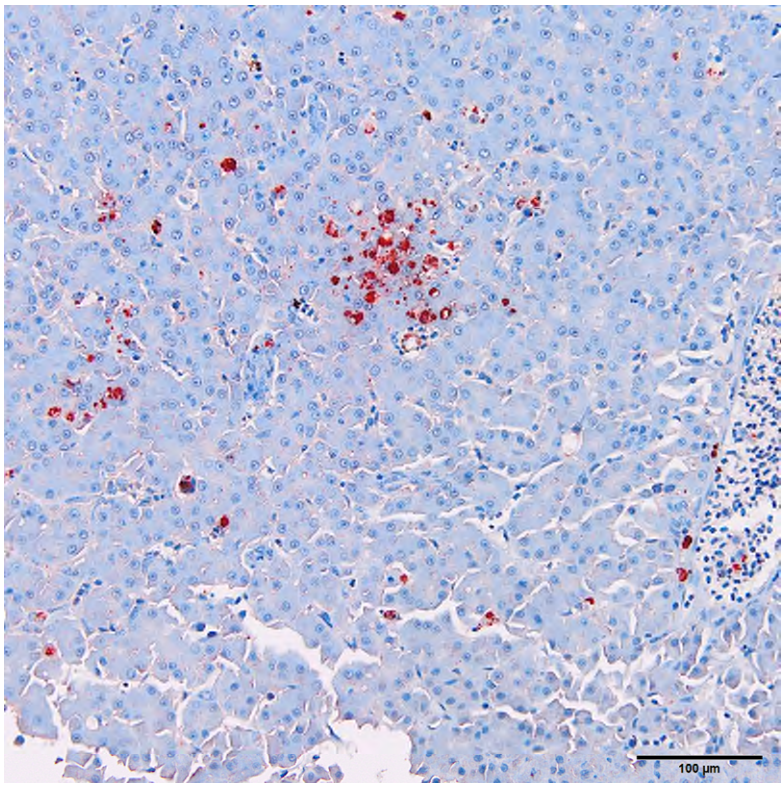
Non-grainy, non-focal, pale, pinkish staining may occur on healthy tissue and is due to non-specific binding.

Figure 1 Immunohistochemistry of Tasmanian RLO-infected fish



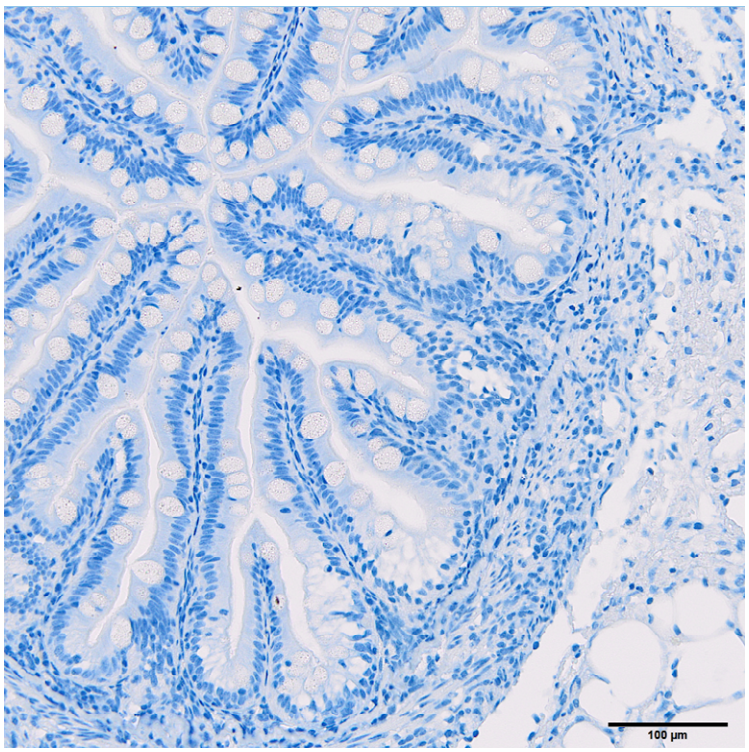
Note: Pyloric caecae/pancreatic tissue, using the anti-*P. salmonis* mouse monoclonal antibody. Magnification ×20.

Figure 2 Immunohistochemistry of *P. salmonis* infected fish



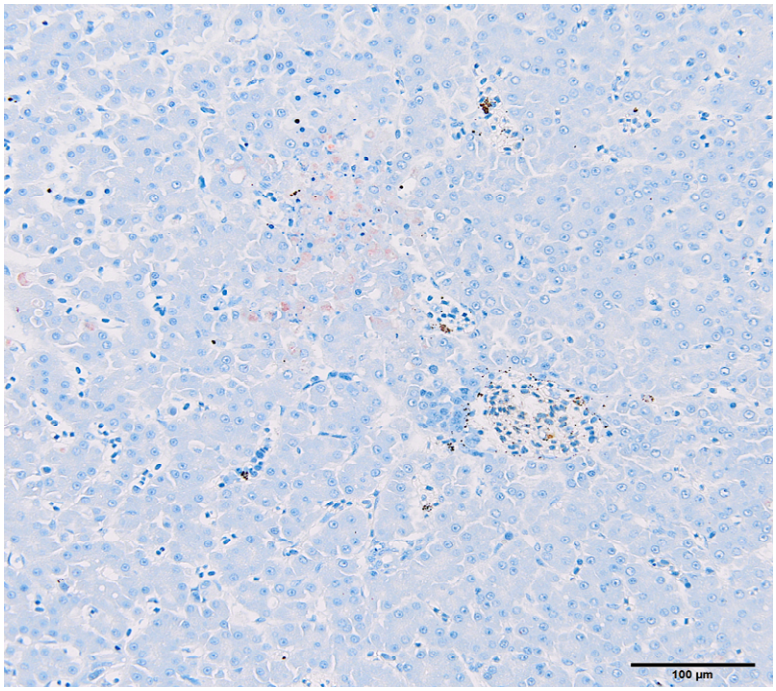
Note: Liver tissue using the anti-*P. salmonis* mouse monoclonal antibody. Magnification ×20.

Figure 3 Immunohistochemistry of Tasmanian RLO-infected fish



Note: Pyloric caecae/pancreatic tissue, using a non-specific monoclonal antibody (anti-White Spot Syndrome Virus). Magnification ×20.

Figure 4 Immunohistochemistry of *P. salmonis* infected fish



Note: Liver tissue using a non-specific monoclonal antibody (anti-White Spot Syndrome Virus). Magnification ×20.

Identification by PCR and amplicon sequencing

Introduction

Both nested (Mauel et al., 1996) and single step (Marshall et al., 1998) PCR assays were developed during the 1990s to facilitate the detection and characterisation of *P. salmonis*. The nested PCR is a sensitive method for detection and identification of *P. salmonis*. Single step PCR methods are also used for detection and identification of *P. salmonis*, but the diagnosticians who developed these methods suggest that the targeted ITS region of the rRNA operon is more variable than the16S region exploited in the nested PCR therefore allowing finer discrimination in the description of new *P. salmonis* isolates.

More recently a quantitative real-time PCR assay for *P. salmonis* was developed (Corbeil et al., 2003) and was shown to be as sensitive as the nested PCR assay. This assay offers the advantages of being faster to perform as well as not presenting the risk of cross-contamination inherent to nested PCR assays. In addition, the real-time PCR allows quantification of *P. salmonis* in samples. Laboratories undertaking these assays will need to verify performance to take into account differences in thermal cyclers.

List of equipment

Apart from the normal range of equipment required in the standard diagnostic laboratory (including refrigerators, freezers, mixers, micropipettes, biological safety cabinets, centrifuges, balances, microwave oven, thermometers), specialised equipment required to undertake diagnostic PCR may include dry heat blocks, thermal cycler, gel electrophoresis equipment, UV transilluminator, and a camera system. The DNA amplicon will need to be sequenced for confirmation of *P. salmonis* gene sequence, however, sequencing services are available at various locations around Australia if sequencing equipment is not available on site.

Reagents

Stored at –20°C

- Taq polymerase
- dNTPs (1.25 mM)
- Mg free buffer 10×
- $MgCl_2$ (25 mM)
- 100% Ethanol AR grade
- 70% Ethanol
- Primers (18 µM)
- 23S FAM probe
- HotStarTaq Mastermix
- 100 bp DNA ladder & loading dye
- TaqMan®Universal Master Mix (qPCR) (Applied Biosystems catalogue number 4304437)
- 18S endogenous control primers and VIC probe (Applied Biosystems catalogue number 4308329 or equivalent)

Stored at room temperature

- QIAamp DNA Mini Kit (QIAGEN)
  - DNA columns
  - Buffer ATL
  - Buffer AL
  - Buffer AW1
  - Buffer AW2
  - Buffer AE
- Proteinase K
- Agarose
- SYBR Safe™ (Life Technology)
- 40 × Tris-Acetate EDTA Buffer

Quality control

Molecular diagnostics 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. In addition, amplification of the 18S rDNA gene can be performed on the DNA extracted from fish tissues to validate the extraction procedure as well as to rule out the presence of PCR inhibitors.

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, that is, not pre-exposed to aquatic pathogens. Wherever possible, it is recommended that disposable containers are used.

Samples should be handled and processed using sterile disposable single-use containers, instruments and reagents to minimize 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.

For each fish sample, approximately 20 mg of inner liver tissues are harvested using sterile scissors and forceps and placed in a sterile 1.5 mL centrifuge tube (conical bottom). Tissues are mashed using a disposable plastic pestle and 180 µL of ATL buffer is added to each sample.

Nucleic acids are extracted from submitted samples in a Biological Safety Cabinet Class II working within a dedicated PCR suite.

Nucleic acid extraction

Piscirickettsia salmonis nucleic acid is obtained from cell culture supernatant or tissue samples using the QIAamp DNA extraction kit following manufacturer's instructions.

Piscirickettsia salmonis-specific nested PCR

Mauel et al., 1996.

Primary amplification

The PCR mixture for a single sample consists of the following reagents:

- 9.5 µL of nuclease-free sterile water
- 12.5 µL of HotStar Taq Master mix
- 0.5 µL of forward primer Eub-B (18 µM) (Table 2)
- 0.5 µL of reverse primer Eub-A (18 µM) (Table 2)
- 2 µL of extracted DNA

Table 2 Primer sequences used in primary amplication of the nested PCR

Primers	Sequence
Eub B forward	AGAGTTTGATCMTGGCTCAG
Eub A reverse	AAGGAGGTGATCCANCCRCA

Note: Eubacterial 16S rDNA universal nested primers.

For multiple samples, the volumes are increased by the appropriate multiple. 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).

Alternatively, the following reagents can be used (per sample) for the primary amplification:

- 12.5 µL of nuclease-free sterile water
- 4 µL dNTPs
- 2.5 µL 10× MgCl2-free buffer
- 1.5 µL MgCl2
- 1 µL Eub-B primer (18 µM) (Table 2)
- 1 µL Eub-A primer (18 µM) (Table 2)
- 0.5 µL Taq polymerase
- 2 µL 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.

To validate the DNA extraction process and rule out the presence of PCR inhibitors in samples the amplification of the host 18S rDNA is performed using the primers in Table 3 and the following cycling conditions:

One cycle at 94°C for 2 minutes (non-HotStar Taq polymerase) followed by 35 cycles of 94°C for 30 seconds (denaturation), 60°C for 30 seconds (annealing) and 72°C for 30 seconds (extension); and, finally, one cycle at 72°C for 5 minutes (final extension).

Amplified DNA (size: approximately 190 bp) is detected by electrophoresis on a 2% (w/v) agarose gel containing 0.5 µg/mL ethidium bromide.

Table 3 Primer sequences used to validate the DNA extraction

Primers	Sequence
18S forward primer	5'- CGG CTA CCA CAT CCA AGG AA -3'
18S reverse primer	5'- GCT GGA ATT ACC GCG GCT -3'

Nested PCR amplification

The second amplification is performed by adding 2 µL of the first PCR products to the reaction mixtures described above except for the presence of the *P. salmonis*-specific primers PS2S and PS2AS (Table 4) instead of Eub-A and Eub-B (Table 2), under the following reaction conditions:

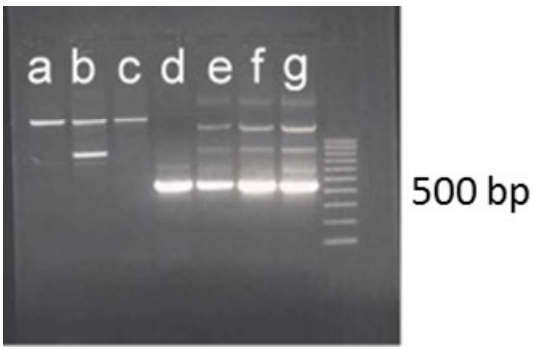
One cycle at 94°C for 15 minutes (if using HotStar Taq, otherwise 1 cycle at 94°C for 2 minutes for non-HotStar Taq); 35 cycles at 94°C for 1 minute, 61°C for 2 minutes and 72°C for 3 minutes; and, finally, one cycle at 72°C for 7 minutes.

Table 4 Primer sequences used in second amplication of the nested PCR

Primers	Sequence
PS2S forward	CTAGGAGATGAGCCCGCTTG
PS2AS reverse	GCTACACCTGCGAAACCACTT

Amplified DNA (size: 469 bp) is detected by electrophoresis on a 2% (w/v) agarose gel containing 0.5 µg/mL ethidium bromide (Figure 5). The Tasmanian and New Zealand RLO are detected by the PCR assay but sequencing of the amplicon reveals sequence differences (Corbeil et al. 2005).

Figure 5 The *P. salmonis*/RLO nested PCR



Note: Amplicons (bright bands) of 469 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 *P. salmonis* isolates LF-89T, ATL-4-91, and NOR-92, respectively. Lane d contained a DNA sample extracted from Tasmanian RLO-infected fish tissue. 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. 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. Note that the Tasmanian and New Zealand RLO are detected by the PCR assay but sequencing of the amplicon reveals sequence differences (Corbeil et al., 2005).

Interpretation

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

- All negative control samples must have no evidence of specific amplicon.
- A positive control sample must yield a specific P. salmonis amplicon of 469 bp.

Amplicons of the correct size are then eluted from the gel, and both strands of DNA sequences are determined (by using the PCR primers PS2S and PS2AS as sequencing primers [Table 4]). Sequence identity and genotype are determined by a BLASTn search of the GenBank database (AY578984). An assay is valid only when all controls yield the expected results.

Single-step PCR

Marshall et al., 1998.

A single-step PCR can be used to amplify the internal transcribed spacer (ITS) of the rDNA operon of exotic isolates of *P. salmonis*. The specific *P. salmonis* primers ITS-1 and ITS-4 (Table 5) are used at a final concentration of 1 µM. Volumes (2 µL) of DNA sample are added to 23 µL of reaction mixture for the amplification. Thermal cycling conditions for the PCR are: one cycle at 95°C for 15 minutes, followed by 35 cycles at 94°C for 30 seconds, 50°C for 30 seconds and 72°C for 30 seconds, followed by one cycle at 72°C for 5 minutes.

Table 5 Primer sequences for *P. salmonis* specific primers

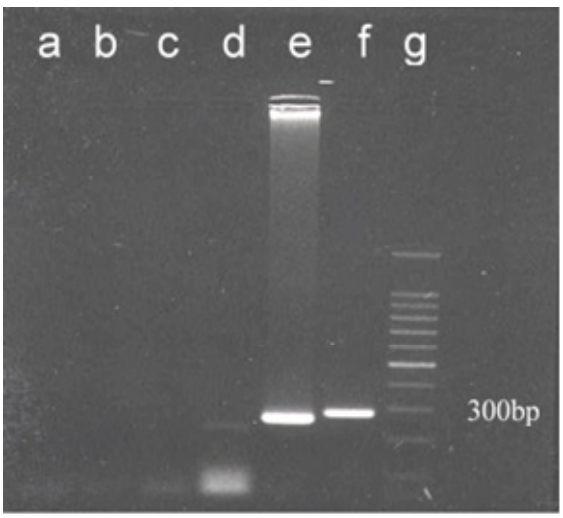
Primers	Sequence
ITS-1	5' TGATTTTATTGTTTAGTGAGAATGA-3'
ITS-4	5'-ATGCACTTATTCACTTGATCATA-3'

PCR products are detected by electrophoresis on a 2% (w/v) agarose gel containing 0.5 µg/mL ethidium bromide or equivalent,for example Gelred. (Figure 6). Sequence identity and genotype are determined by a BLASTn search of the GenBank database (accession number AY578985).

This PCR will also amplify the Tasmanian and New Zealand RLO DNA. The Tasmanian RLO and NZ-RLO1 amplicon has a 19 bp deletion towards the 3'-end of the amplicon (Corbeil et al., 2005). A BLASTn search will also confirm the identity of the amplicon.

Running both *P. salmonis* and the Tasmanian RLO DNA or NZ-RLO1, on the 2% agarose gel as positive controls can facilitate interpretation of the results as it is usually possible to visually differentiate the amplicons based on size (migration in the gel). Sequence analysis must always be undertaken for confirmation.

Figure 6 Detection of *P. salmonis* using a single-step PCR method



Note: The primers used for the rDNA amplification were RTS-1 and RTS-4 (Mauel et al., 1996) producing a 283 bp amplicon in *P. salmonis*. Several other species of bacteria were used as specificity controls. Lanes: **a** *Hafnia alvei*, **b** *Aeromonas salmonicida*, **c** *Yersinia ruckeri*, **d** *Vibrio anguillarum*, **e** Tasmanian RLO, **f** *P. salmonis* (ATL-4-91), **g** 100 bp ladder. *Vibrio anguillarum* shows a weak non-specific band just below the expected *P. salmonis* amplicon. Note that the Tasmanian RLO is detected by the PCR assay but has a slightly smaller amplicon than that amplified using *P. salmonis* (ATL-4-91) gDNA. In addition, sequencing revealed that the amplicon from the Tasmanian RLO is shorter than the amplicon from *P. salmonis* by 19 base pairs (Corbeil ert al., 2005).

Real-time qPCR (TaqMan assay)

Corbeil et al., 2003.

The quantitative real-time PCR (qPCR) can be used to evaluate the titre of *P. salmonis* in fish tissues (Figure 7). In order to do so, a dilution series of DNA extracted from *P. salmonis* titrated in cell culture must be used to establish a reference curve (Marshall et al., 1998). Alternatively, the real-time PCR assay, which was shown to be as sensitive as the nested PCR assay (Marshall et al., 1998), can also be used simply as a qualitative assay. The qPCR assay has the advantages that it is rapid,

quantitative, and less prone to cross-contamination, which can occur with conventional nested-PCR assays.

Primers and probe for the TaqMan assay were designed using Primer Express Software version 1.5 (PE Applied Biosystems). The *P. salmonis* primers and probe are based on the 23S rDNA gene, a relatively conserved genomic region among several isolates of *P. salmonis*. Primer and probe sequences are shown in Table 6.

Table 6 Primer and probe sequences for the TaqMan assay

Primers	Sequence
Forward primer (F-760)	5'-TCTGGGAAGTGTGGCGATAGA-3'
Reverse primer (R-836)	5'-TCCCGACCTACTCTTGTTTCATC-3'
Labelled probe (PS23S)[a]	6FAM-TGATAGCCCCGTACACGAAACGGCATA-TAMRA

a 6-carboxyfluorescein (FAM) and 6-carboxytetramethylrhodamine (TAMRA).

The *P. salmonis* primers are used at a final concentration of 900 nM. The PS23S FAM probe is used at a final concentration of 250 nM.

The 18S rRNA endogenous control primers and probe are used to validate the DNA extraction procedure from fish tissues, confirm the integrity of the extracted DNA and determine the absence of PCR inhibitor (Table 7).

Table 7 Primer and probe sequences of 18S rRNA endogenous controls

Primers	Sequence
18S forward primer	5'-CGGCTACCACATCCAAGGAA-3'
18S reverse primer	5'-GCTGGAATTACCGCGGCT-3'
18S probe	VIC-TGCTGGCACCAGACTTGCCCTC-TAMRA

Note: used to validate the DNA extraction procedure from fish tissues, confirm the integrity of the extracted DNA and determine the absence of PCR inhibitor.

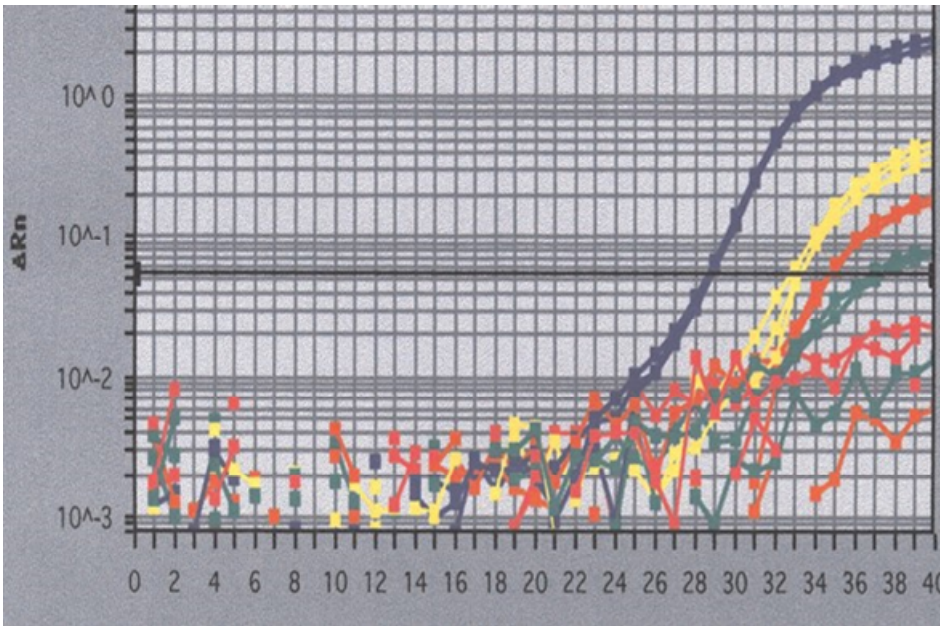
The primers are used at a limiting concentration of 113 nM each. The VIC probe is used at a final concentration of 31 nM. The reactions are carried out in a 96-well plate in a 25 µL reaction volume containing 12.5 µL Universal Master Mix (PE Applied Biosystems). Volumes (2 µL) of each DNA sample are added. Standard thermal cycling conditions are used (50°C for 2 minutes, 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute).

A sample is considered positive when the change in fluorescence (ΔRn) of FAM or VIC, relative to that of ROX (internal reference signal), exceeds the set threshold values of 0.055 for FAM and VIC in the linear range of the amplification plots at a cycle threshold (CT) value below 40. CT is defined as the cycle at which a statistically significant increase in fluorescence output above background is detected.

It should be noted that the TaqMan assay detects *P. salmonis*, Tasmanian RLO and the New Zealand RLOs. To confirm the identity of the aetiological agent, one of the conventional PCR tests and sequencing of the amplicon must be performed.

New Zealand has developed strain specific real-time PCR assays for NZ-RLO1 and NZ-RLO2 (unpublished).

Figure 7 *P. salmonis* qPCR assay



Note: The graph shows the log of the change in fluorescence (y-axis) intensity versus the number of amplification 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; orange curve: 1 $TCID_{50}$/mL; green curve: 0.5 $TCID_{50}$/mL; reddish 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 the 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.

Piscirickettsia salmonis and Tasmanian rickettsia-like organism (TRLO) culture

Introduction

Piscirickettsia salmonis can be grown on solid agar media such as BCG (Mauel et al., 2008), CHAB (Mikalsen et al., 2008), Austral-TSFe (Yanez et al., 2013), Austral-TSHem (Yanez et al., 2013) and SRS-BA (OIE, 2016) agar, as well as in cell culture. Media are inoculated according to standard microbiological techniques and the identity of colonies confirmed as *P. salmonis* using supplementary diagnostic tests such as PCR or qPCR (see above). While these bacteriological media are available, complete diagnostic methods are yet to be optimized. Further, it is not known whether these media are more or less efficient in the recovery of *P. salmonis* in culture than the cell culture isolation protocol described in early editions of the OIE Aquatic Manual (Brosnahan et al., 2017).

The Tasmanian RLO is a fastidious bacterium and can be grown on specialised enriched media such as BCG agar Mauel et al., 2008). BCG agar was originally developed for a *Francisella* sp. from cod56 and is also suitable for the growth of the exotic *P. salmonis* (Mauel et al., 2008). Tasmanian RLO and NZ-RLO1 can also be grown on CHAB agar (Mikalsen et al., 2008) and in cell culture using the CHSE-214 cell line. However, solid medium culture is preferred over cell culture as it does not sustain viral replication and it is therefore potentially more specific.

NZ-RLO2 cannot be grown on CHAB, BCG or BCGT agar and the growth characteristics of NZ-RLO3 are unknown.

List of equipment

- Autoclave
- Refrigerated incubator
- Sterile specimen containers
- Sterile sampling devices

Reagents

Refer to Appendix A: Reagents and kits.

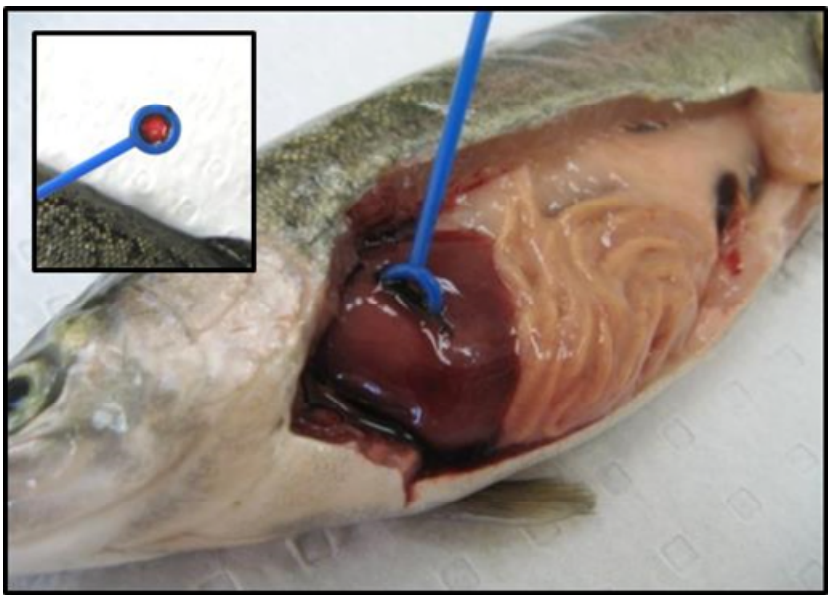
Quality control

Microbiological testing procedures should be accredited for compliance with ISO/IEC 17025.

Procedure

Using aseptic technique, samples should be obtained directly from the liver (Figure 8). A sterile 10 µl disposable loop is ideal for sampling (Figure 8).

Figure 8 Sampling for Tasmanian RLO

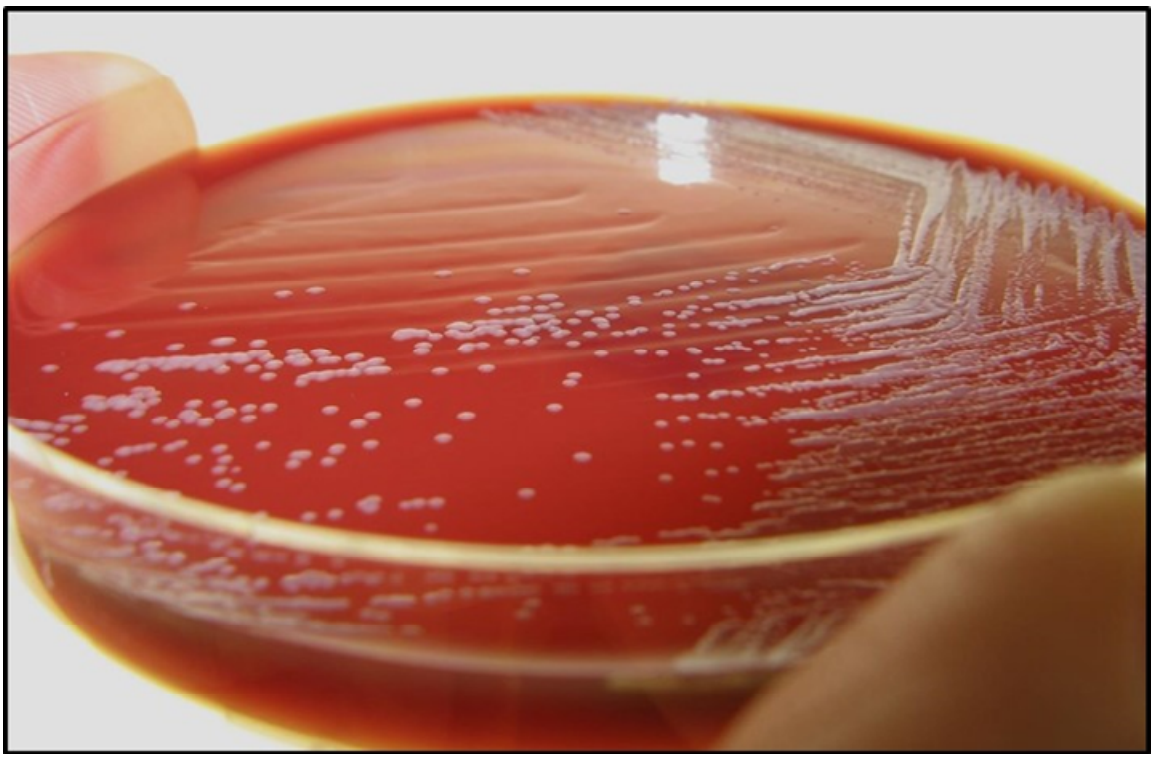


Note: To sample for Tasmanian RLO, take a sterile disposable plastic loop, plunge the eye of the loop into the liver and rotate the loop once or twice. Carefully withdraw the loop and inoculate a plate of BCG. Photos courtesy Dr J. Carson.

Agar plates should be incubated in air at 22°C for up to 7 days. If secondary infection with other bacteria is suspected, samples should be cultured on BCG agar supplemented with trimethoprim (BCGT agar; Appendix A: Reagents and kits).

Small Tasmanian RLO colonies can be observed after 72 hours at 22°C and continue to grow with additional incubation (Figure 9). Tasmanian RLO (TRLO)colonies are white to beige, opaque, circular in form, convex, with an entire to undulate margin. Colony consistency is viscous. TRLO are Gram-negative, non-motile, pleomorphic coccoid-shaped bacteria of approximately 1.5 µm in diameter.

Figure 9 Tasmanian RLO on BCG agar



Note: After 5 days incubation at 22°C.

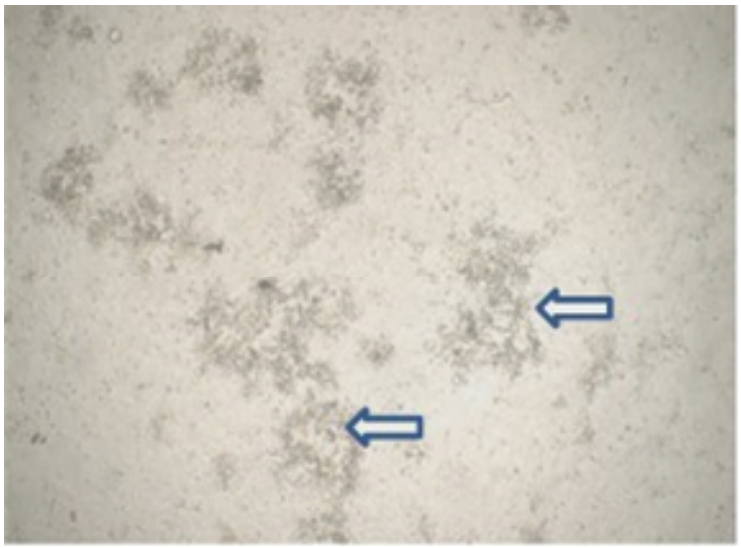
Tasmanian RLO cultures can be presumptively identified using qPCR (Corbeil et al., 2003) and confirmed by sequence analysis of the 16S-23S internally transcribed spacer (ITS) region using the PCR amplification method of Mauel et al., (1996) or the single step PCR described in the PCR method described in the previous section. Tasmanian RLO ITS sequences feature a 19 bp deletion, which distinguishes the bacterium from *P. salmonis* (Corbeil et al., 2005).

Isolation of *Piscirickettsia salmonis* in cell culture

Introduction

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 cell lines were shown to be susceptible (Figure 10) with CHSE-214 being the most susceptible followed by BF-2.

Figure 10 Growth of *P. salmonis* in cell culture



Note: Photomicrograph of the cytopathic effect (CPE; arrows), typical of *P. salmonis*, developing in cell cultures of CHSE-214 cell line. CHSE-214 cultures at day 11 after infection with *P. salmonis* NOR-92 strain (Norway) in the presence of 100 µg/mL ampicillin. 100 µL of a 104 $TCID_{50}$/mL stock suspension of *P. salmonis* was used to infect cells. The CHSE-214 and EPC cell lines are also susceptible to the Tasmanian RLOs (results not shown), other cell lines are yet to be tested.

List of equipment

Normal range of equipment required in the standard diagnostic laboratory. For example:

- refrigerators
- freezers
- mixers
- micropipettes
- biological safety cabinets
- centrifuges
- microscope

Reagents

- CHSE-214 cell line
- Eagle's minimum essential medium (EMEM)
- 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) 1 M
- Fetal Calf Serum (FCS) 10%
- Glutamine 1X
- CO^2 5%
- Balance Salt Solution (BSS)

Quality control

Cell culture diagnosis should be operated under an ISO 17025 accredited and audited quality assurance program. The pathogen identification is performed via PCR and sequencing. It is suggested that, if available, other PCRs be run for the presence of other pathogens endemic to the region (for example in Tasmania: TSRV, TABV, POMV). Alternatively, confirmation can be done on BCG agar as it does not sustain viral replication and it is therefore potentially more specific.

Procedure

Preparation of tissue

1) The kidney must be aseptically removed and transferred to a sterile container. Antibiotics must not be used in the isolation procedure. Tissues must be kept at 4°C or on ice until processed, and must not be frozen.
2) Kidney tissue is homogenized at 1/20 in antibiotic-free balanced salt solution (BSS), and then, without centrifugation, further diluted 1/5 and 1/50 in antibiotic- free BSS for inoculation on to cell cultures. Final dilutions for use are 10^{-2} and 10^{-3}.

Inoculation of cell monolayers

1) A 10^{-2} and 10^{-3} dilution of the organ homogenates is inoculated onto cultured cell monolayers and maintained in antibiotic-free medium.
2) The diluted homogenate can be inoculated directly (0.1 ml/culture) into the antibiotic-free cultured medium overlaying the cells.
3) The cell cultures must be incubated at 15°C to 18°C for 28 days and observed for the appearance of a cytopathic effect (CPE). The *P. salmonis* CPE consists of plaque-like clusters (Figure 10). With time, the CPE progresses until the entire cell sheet is destroyed.
4) If CPE does not occur (except in positive controls), cultures should be incubated for an additional 14 days.

Experiments carried out at AAHL showed that ampicillin used at a concentration of 100 µg/mL of culture medium did not inhibit the growth of *P. salmonis*. Preliminary testing of several TRLO south east serotype isolates by Dr Jeremy Carson indicates that they are very susceptible to ampicillin. TRLO isolates from the Tamar River and Macquarie Harbour are yet to be tested.

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Appendix A: Reagents and kits

Nylund et al., 2006; Otterlei et al., 2016.

BCG agar

1) Dissolve the ingredients (Table 8).
2) Check pH and adjust if necessary to within a range of 6.7 to 6.8.
3) Sterilise by autoclaving at 121°C for 15 minutes.
4) Cool to 50°C in a water bath.
5) Aseptically add 5% of defibrinated sheep blood and mix by gentle swirling.
6) Pour as plates.

Table 8 Base ingredients required for BCG agar

Ingredient	Amount
Blood agar base medium (Oxoid Blood Agar Base No.2 CM271)	40.0 g
L-cysteine	1.0 g
D-glucose	10.0 g
Distilled water	1000 mL

Selective BCGT agar

7) Dissolve the ingredients (Table 9).
8) Check pH and adjust if necessary to within a range of 6.7–6.8.
9) Sterilise by autoclaving at 121°C for 15 minutes.
10) Cool to 50°C in a water bath.
11) Aseptically add 5% of defibrinated sheep blood and mix by gentle swirling.
12) Immediately before pouring plates, add 10 mL of 0.2 µm filter-sterilised 0.16% (w/v) trimethoprim and mix by gentle swirling.
13) Pour as plates.

Table 9 Base ingredients required for selective BCGT agar

Ingredient	Amount
Blood agar base medium (Oxoid Blood Agar Base No.2 CM271)	40.0 g
L-cysteine	1.0 g
D-glucose	10.0 g
Distilled water	1000 mL