Broth medium for the successful culture of the fish pathogen *Piscirickettsia salmonis*

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ABSTRACT: *Piscirickettsiosis* or salmonid rickettsial septicaemia (SRS) caused by *Piscirickettsia salmonis* constitutes one of the main problems in farmed salmonid and marine fishes. Since the first reports of the disease, it has been successfully isolated and maintained in eukaryotic cell-culture systems, but these systems are time-consuming, the media are costly, and eliminating heavily contaminated host cell debris is difficult. In this report, we describe a marine-based broth supplemented with L-cysteine, named AUSTRAL-SRS broth, that facilitates superior growth of *P. salmonis* strains. Strains reached an optical density of approximately 1.8 when absorbance was measured at 600 nm after 6 d incubation at 18°C. Several passages (n = 6) did not alter the culture kinetics. We report for the first time the purification of DNA, lipopolysaccharide (LPS) and whole membrane protein obtained from *P. salmonis* grown in this liquid medium, and thus provide a suitable platform to simplify the preparation of *P. salmonis* cells for genetic and serological studies. Moreover, the results of the cytopathic effect test showed that *P. salmonis* grown in AUSTRAL-SRS broth maintained their virulence properties, inducing apoptosis after 3 d. This makes the medium a good candidate for the successful growth of *P. salmonis* and an excellent basis for the development of low cost vaccines.

KEY WORDS: *Piscirickettsia salmonis* · Broth · Culture

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INTRODUCTION

Piscirickettsiosis or salmonid rickettsial septicaemia (SRS) caused by *Piscirickettsia salmonis* constitutes one of the main problems in farmed salmonid and marine fishes (see reviews by Almendras & Fuentealba 1997, Mauel & Miller 2002, Fryer & Hedrick 2003). This fastidious pathogen was initially observed in southern Chile in 1989 (Bravo & Campos 1989, Branson & Diaz-Muñoz 1991, Cvitanich et al. 1991), followed by observations in western Canada (Brocklebank et al. 1992), Ireland (Rodger & Drinan

1993), Scotland (Grant et al. 1996), Norway (Olsen et al. 1997), eastern Canada (Cusack et al. 2002) and southern California, USA (Arkush et al. 2005).

The disease is preliminarily diagnosed by the clinical signs of the affected fish, which usually show a systemic infection targeted to the kidney, liver, spleen, brain, intestine, ovary and gills of the fish (Cvitanich et al. 1991, Garcés et al. 1991). Normally, infected samples are subjected to microscopic and histological observation (Lannan & Fryer 1991), but this diagnosis must be confirmed by the isolation of *Piscirickettsia salmonis* from tissues cultured within

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fish cell lines *in vitro* (Fryer et al. 1990), followed by immunohistochemistry, polymerase chain reaction on DNA extracted from infected tissues, or by subjecting cell-cultured bacteria to a specific fluorescent antibody test identification (OIE 2006). The use of agar media is an alternative that shows potential and would relieve facilities of the cost of maintaining cell lines as these are time-consuming and costly, and it would eliminate heavily contaminated host cell debris. The use of specific enriched sheep blood agar prepared with cysteine has been developed for the cultivation of *P. salmonis* strains (Mauel et al. 2008, Mikalsen et al. 2008). Growth on solid media usually takes from 4 to 6 d for visible colonies to appear, but these are often poorly defined, leading to inaccuracies in estimations of the bacterial concentration.

During the development of a solid medium, Mikalsen et al. (2008) employed cysteine heart broth, with or without 5 % sheep blood, in order to study the growth of one *Piscirickettsia salmonis* isolate, but these authors did not detail the rates and overall levels of growth in broth. Here we describe a marine broth medium supplemented with L-cysteine, named AUSTRAL-SRS broth, that facilitates growth of *P. salmonis* strains. This broth should make it possible to culture strains needed to determine the minimum inhibitory concentration of drugs and to prepare cells for the development of low-cost vaccines for protection against piscirickettsiosis.

MATERIALS AND METHODS

Bacterial strains

Two *Piscirickettsia salmonis* isolates (PPT-05 and PPT-15) recovered in 2008 from diseased farmed rainbow trout *Oncorhynchus mykiss* in Chile were used to test the liquid culture media. The type strain *P. salmonis* ATCC VR-1361 (equivalent to LF-89) originally obtained from coho salmon *O. kisutch* was included for comparative purposes and was obtained from the American Type Culture Collection (ATCC). All bacterial strains were confirmed as *P. salmonis* using PCR-based analysis described by Marshall et al. (1998) and also by an indirect fluorescent antibody test (IFAT, SRS–BiosChile) according to the manufacturer's recommendation. For growth on solid media, bacteria were streaked onto tryptone soy supplemented with ferric nitrate (AUSTRAL-TSFe) agar plates and incubated at 18°C for 10 d. Stock cultures were prepared from cells scraped off TSFe plates and resuspended in 1 ml of L-15 Leibovitz medium con-

taining 20 % fetal bovine serum (AUSTRAL-FBS) and 10 % dimethyl sulphoxide (DMSO) and stored frozen at –80°C or in Criobille tubes (AES Lab).

Liquid culture medium

Initially, 110 different formulations were tested to evaluate the efficacy of growing the *Piscirickettsia salmonis* type strain. In these different media, formulations with several concentrations/combinations of marine broth, sodium chloride, triptone soy, Yeastolate, L-cysteine, ferric salt and hemoglobin were tested. Marine broth was obtained from Becton, Dickinson and Company, L-cysteine and Yeastolate from USBiological, and ferric nitrate from Merck. Only 2 formulations facilitated growth of *P. salmonis*, ATCC VR-1361 being one of them and AUSTRAL-SRS broth, which was the best medium. The composition and preparation of the AUSTRAL-SRS broth is presented in Appendix 1. To determine the optimal concentration of the 3 components, an independent study with each component was preliminary tested using L-cysteine concentrations of 1 to 50 g l⁻¹, and between 0.001 and 1 mM for ferric nitrate. The final pH of the medium was between 6.0 and 6.4 at room temperature. FBS is also a critical component and was added to a final concentration of 5 % p/v.

Incorporation of *Piscirickettsia salmonis* into broth cultures

Five colonies grown on AUSTRAL-TSFe plates were used to prepare the starting inocula of all *Piscirickettsia salmonis* isolates. Bacterial suspensions were prepared in sterile AUSTRAL-SRS broth, and 2 ml aliquots were adjusted at 1.0 absorbance at 600 nm measured on a spectrophotometer. These aliquots were then inoculated into 100 ml Erlenmeyer flasks containing 20 ml culture medium, resulting in an initial bacterial concentration of 10⁸ cells ml⁻¹ as determined by direct microscopy count (equivalent to 10⁷ colony-forming units [CFU] ml⁻¹). Control cultures were run simultaneously without the addition of bacteria. Incubation was carried out for 10 d at a controlled temperature of 18°C, with or without moderate agitation (50 rpm). The light regime used was 0 h light:24 h dark. The experiment was carried out in triplicate for each bacterial strain.

In order to examine whether *Piscirickettsia salmonis* can suffer a reduction in growth, a *P. salmonis* type strain was subjected to 6 serial passages using

as inocula for each passage 2 ml aliquots obtained from the preceding bacterial culture after 6 d of incubation.

Evaluation of bacterial growth curves

To determine the growth of the *Piscirickettsia salmonis* strains, absorbance at 600 nm was recorded every 24 h, and at the same time the evolution of culturable cells in each flask was carried out by seeding serial diluted samples in L-15 medium with 10 % FBS and placing them on AUSTRAL-TSFe agar plates followed by incubation at 18°C for 10 d. All plating was done in duplicate, and the number of viable bacterial cells in the broth culture was calculated from the dilution that yielded between 30 and 300 colonies on the agar plates.

Identification of *Piscirickettsia salmonis* by PCR

Samples (1 ml) from each broth culture were taken daily during the first week and colonies were collected and placed in 1.5 ml sterile tubes, which were then centrifuged at $12\,000 \times g$ for 5 min. Extraction of DNA from bacterial pellets was performed using InstaGene Matrix (Bio-Rad) according to the manufacturer's instructions. The concentration of each DNA sample was adjusted to 10 to $20 \text{ ng } \mu\text{l}^{-1}$, and 1 μl of each DNA solution was used in each amplification reaction. To confirm their designation as *P. salmonis*, all strains were tested using the primer pair RT1–RTS2 designed by Marshall et al. (1998). The amplification cycles used for denaturation, primer annealing and extension were carried out according to a published PCR protocol (Marshall et al. 1998). A positive result was indicated by the presence of an amplification product of 91 bp after electrophoresis through a 1.5 % (w/v) agarose gel. All analyses were carried out with DNA obtained in 2 different extractions.

Analysis of cellular changes and surface components

To determine whether *Piscirickettsia salmonis* suffered cellular changes during the experimental period, samples of bacterial culture were visualised every day by light microscopy. Moreover, we analyzed the lipopolysaccharide (LPS) and membrane protein profiles due to possible changes in the com-

ponent of the bacterial cell wall. LPSs from the cell envelopes were obtained by the Proteinase K (PK) method following the procedures of Hitchcock & Brown (1983). Samples were examined by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE; Laemmli 1970), with 14 % (w/v) acrylamide in the resolving gel and 4 % (w/v) acrylamide in the stacking gel, and then silver stained following the procedure of Tsai & Frasch (1982). The whole cell envelope proteins from the *P. salmonis* strains were prepared from bacterial culture grown in AUSTRAL-SRS broth as described by Pot et al. (1994) and examined by SDS-PAGE with 12 % (w/v) acrylamide in the resolving gel and 4 % (w/v) acrylamide in the stacking gel. Protein bands were stained with 0.05 % Coomassie Brilliant Blue R (Sigma), photographed, and the relative mobilities of each were determined by comparison with standard protein markers.

Evaluating *Piscirickettsia salmonis* infectivity *in vitro*

The invasion assays were performed in salmon head kidney (SHK-1) cells cultured at 18°C in 24-well tissue culture plates. A concentration of 1×10^5 SHK 1 cells was inoculated into each well and grown in Leibovitz's L-15 medium supplemented with 10 % SFB, 6 mM L-glutamine (Hyclone Laboratories) and 40 μM 2-mercaptoethanol (Gibco, Invitrogen Laboratories). One day after incubation, 1000 μl of *P. salmonis* suspension prepared in L-15 medium with 2 % FBS (1×10^6 cells well^{-1}) were inoculated into each well containing the cell monolayers and incubated at 18°C for 9 d. Control cell cultures were run simultaneously without the addition of bacteria.

Possible differences in the permeability of the membrane in SHK-1 cells exposed to the *Piscirickettsia salmonis* were determined by evaluating the liberation level of the cytosolic enzyme lactate dehydrogenase (LDH) in the cell supernatant using the commercial LDH-P UV Assay (Wiener Lab) kit. In brief, samples of 1 ml of cell culture, taken daily, were centrifuged at 1600 rpm ($176 \times g$) for 5 min, and the LDH liberation level in 20 μl of supernatant was obtained in accordance with the supplier's instructions. Absorbance at 340 nm was read after 30, 60, 120 and 180 s from the first reading. The average difference of the absorbance ($\Delta A \text{ min}^{-1}$) was determined by subtracting each absorbance value from 2 replicates and averaging the LDH. The result was obtained using the equation: $\text{LDH (IU l}^{-1}\text{)} = \Delta A \text{ min}^{-1} \times \text{factor (8.095)}$.

In addition, the morphology of the fish cell line was examined using a phase-contrast inverted microscope at 400 or 600 $\times$ magnification.

Statistical analyses

The results were subjected to an analysis of variance (ANOVA; Sokal & Rohlf 1980) in order to compare growth among treatments with or without agitation and in control culture. An alpha value of $p < 0.05$ was used for all analysis.

RESULTS AND DISCUSSION

Before the liquid medium was used to culture all *Piscirickettsia salmonis*, a total of 110 specific marine or triptone soy broth formulations were screened by inoculating the *P. salmonis* type strain directly into each liquid medium. Only 2 of the 110 formulations tested showed growth at different levels, and the medium named AUSTRAL-SRS broth generated the better growth compared to the other media (Fig. 1), while the remaining 108 formulations did not support the growth of this strain.

In each medium, the *Piscirickettsia salmonis* type strain was identified by PCR analysis and IFAT tests and confirmed by partial 16S rRNA gene sequencing (data not shown). AUSTRAL-SRS broth was selected as ideal candidate for further studies. According to the criteria described by McGann et al. (2010), a liquid medium must induce rapid, high-density bacterial growth as measured by optical density (OD) at 600 nm and promote the efficient growth of low bacterial inocula.

When the growth curves of all *Piscirickettsia salmonis* were determined by measuring absorbance, the 3 strains grew well with and without moderate agitation (Fig. 2A). However, statistical analysis revealed a significant difference ($p < 0.05$) in the growth of all *P. salmonis* that were dependent on the movement of the broth culture. Maximum cell density was obtained with agitation (50 rpm), with 64.7 % more than the density obtained in the static Erlenmeyer flasks after 6 d of incubation. In fact, the growth of *P. salmonis* with gentle shaking was characterised by a short lag phase (approximately 18 h) followed by rapid logarithmic growth, achieving a maximal OD of approximately 1.8 after 6 d of incubation. From this point on, growth remained nearly constant during the experimental period (10 d) at OD values of approximately 1.68.

Fig. 1. *Piscirickettsia salmonis*. Comparison of the growth ($\pm$ SD) of the type strain in 2 of the 110 specific marine or triptone soy broth formulations tested in this study. TSFe broth: tryptone soy supplemented with ferric nitrate. Growth tests performed with *P. salmonis* on the remaining 108 media did not support the growth of the type strain after 10 d of incubation at 18°C

It is important to point out that no difference was observed in the growth dynamics between the 2 *Piscirickettsia salmonis* isolates and the type strain, regardless of the number of replicates and the agitation condition, suggesting that in AUSTRAL-SRS

Fig. 2. *Piscirickettsia salmonis*. (A) Average growth of all strains used in this study in AUSTRAL-SRS broth with or without agitation and (B) the concentration of viable bacteria reached with agitation. The control was a liquid culture without the addition of bacteria. CFU: colony-forming unit. Vertical lines show $\pm$ SD

broth, the behaviour of this bacterium is reproducible. In fact, when a serial passage experiment was carried out, the *P. salmonis* type strain displayed similar growth kinetics, regardless of the number of passages, reaching absorbance values between 1.7 and 1.8 (data not shown).

Although the *Piscirickettsia salmonis* strain grew on AUSTRAL-TSFe agar, poorly defined colonies were produced, leading to inaccuracies in estimations of the CFU concentration. Thus, the bacterial count showed that the number of culturable bacteria during the first 5 d decreased by 4 log-units from an initial inoculum of 10^7 CFU ml⁻¹ (equivalent to 10^8 cells ml⁻¹). After this period, the number of culturable bacteria increased 2 log units of CFU ml⁻¹ at the end of the experiment, allowing the detection of 10^5 *P. salmonis* CFU ml⁻¹ (Fig. 2B).

Given that the persistence of culturable cells was lower than expected in correlation to absorbance values, we applied quantitative real-time PCR (qRT-PCR) designed by Karatas et al. (2008) (data not shown). A slight increase in the number of *Piscirickettsia salmonis* cells from the initial inoculum was determined during the first 6 d (data not shown). Then, 12 d after the beginning of the experiment, the qRT-PCR count showed that the number of bacteria increased by 1 log unit, reaching 7.3×10^9 cells. Therefore, *P. salmonis* lost its ability to grow on solid media, but retained viability in liquid media.

Despite the fact that the *Piscirickettsia salmonis* strains grew on specific enriched sheep blood agar prepared with cysteine (Mauel et al. 2008, Mikalsen et al. 2008) as well as in the blood-free medium employed in this work, the efficiency of recovering *P. salmonis* from all media is not suitable for determining the concentration of the bacterium, mainly because bacterial growth is extremely slow. In fact, growth on solid media usually takes from 4 to 8 d for visible colonies to appear (Mauel et al. 2008, Mikalsen et al. 2008). This disadvantage leads us to express the growth of *P. salmonis* in absorbance units or cells.

Microscopic observations under phase contrast of *Piscirickettsia salmonis* cultured into AUSTRAL-SRS broth did not reveal changes in the morphology and size of the bacteria, which appeared as Gram-negative cocci. An aliquot of each culture was observed under an epifluorescence microscope every day in order to confirm the presence of *P. salmonis* when exposed to a commercial rabbit fluorescein isothiocyanate (FITC)-conjugated anti-*P. salmonis* antibody. A strong and positive specific reaction was shown. As expected, liquid culture without the addition of *P. salmonis* did not yield any growth.

The result of the LPS profiling showed that all Chilean *Piscirickettsia salmonis* isolates and the type strain displayed a similar LPS pattern with a ladder of low molecular weight (LMW) O-antigen bands, but less abundant high molecular weight (HMW) species were present (Fig. 3A), matching the typical profile of the *P. salmonis* species reported by Kuzyk et al. (1996). These authors noted that silver staining of PK-digested *P. salmonis* revealed a ladder-like banding pattern of carbohydrates ranging from 16 to 35 kDa in size with a discrete band around 20 kDa and an intensely stained major band around 11 kDa. It is important to note that Kuzyk et al. (1996), using rabbit polyclonal antibodies, recognized this band as an 11 kDa carbohydrate antigen corresponding to a lipooligosaccharide component of LPS, which was confirmed by other studies (Barnes et al. 1998, Jamett et al. 2001).

With regard to the analysis of whole cell envelope proteins, all Chilean *Piscirickettsia salmonis* strains presented a similar profile, displaying a considerable number of common bands between 116 and 25 kDa (Fig. 3B). Similar results were reported by Barnes et al. (1998), who noted that *P. salmonis* studied contained a large, apparently similar protein, with 5 major protein bands with molecular masses of 30, 42, 58, 70 and 105 kDa.

Adherence, invasion and intracellular replication in the host cells are important for pathogenesis by intracellular pathogens (Finlay & Falkow 1997, Nobbs et al. 2009). It is well known that *Piscirickettsia salmonis* replicates by binary fission within membrane-bound cytoplasmic vacuoles in cells of susceptible fish hosts or fish cell lines inducing a characteristic cytopathic effect (CPE; Fryer & Hedrick 2003).

Many studies on *Piscirickettsia salmonis* consider that Chinook salmon embryo (CHSE-214) cells offer considerable advantages over other fish, insect and frog tissue culture cells. However, Birkbeck et al. (2004) showed that *P. salmonis* replicates in higher titers in an insect cell line than in the CHSE-214 cells that are normally used to culture the organism and that *P. salmonis* retains virulence for Atlantic salmon *Salmo salar* after repeated culture in insect cells. On the other hand, the tissue chosen for the isolation of *P. salmonis* during active infection in salmonids is kidney, and SHK-1 is a cell line from *S. salar* head kidney, which exhibits macrophage properties (Dannevig et al. 1997).

Unpublished work in our laboratory has shown that *Piscirickettsia salmonis* strains produced CPE in CHSE-214 cells similar to that of SHK-1 cells,

Fig. 3. *Piscirickettsia salmonis*. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) of (A) silver-stains of lipopolysaccharides and (B) Coomassie stains of whole cell envelope proteins from Chilean isolates and the type strain cultivated in AUSTRAL-SRS broth. Lanes: MW, molecular weight markers; 1, the type strain LF-89^T; 2 and 3, the Chilean isolates PPT-005 and PPT-0015, respectively. Numbers on the left indicate the positions of MWs (kDa)

Fig. 4. (A) Lactate dehydrogenase (LDH) liberation (mean $\pm$ SD) in salmon head kidney (SHK-1) cells infected with *Piscirickettsia salmonis* and control (non-infected) cells over 9 d. (B) The cytopathic effect on SHK-1 cells incubated with *P. salmonis* for 2, 5 and 8 d. No structural changes were detected in SHK-1 cells without *P. salmonis* (control) (data not shown)

although the cell sheet was completely lysed 15 d post infection. Therefore, we decided to evaluate the effect of *P. salmonis* cells cultured in AUSTRAL-SRS broth into the SHK-1 cell line. Microscopic analysis revealed infectivity patterns similar to those reported by Olavarria et al. (2010), who observed the CPE after 3 d post infection, and due to their growth conditions, the infection spread to neighboring cells with total degenerative changes occurring in the SHK-1 cell line after 5 d of incubation (Fig. 4B).

In addition, the CPE was accompanied by a significant increase in the level of LDH liberation in SHK-1 cells inoculated with *Piscirickettsia salmonis* from Day 2 to Day 5 of the study, when *P. salmonis* showed the greatest level of liberation owing to greater damage of the cell membrane (Fig. 4A). After this, LDH analyses showed that the values declined very rapidly during the first 24 h, followed by a stabilization around 70 LDH IU l⁻¹ until the end of the experiment.

As expected for the monolayers not inoculated with *Piscirickettsia salmonis* isolates, negative results of the CPE test and LDH analysis were found. Further confirmation of the identity of *P. salmonis* isolates was made using the IFAT test.

Our results suggest that, in addition to having the capacity to establish the infectious cycle and overcome cell barriers, this bacterium may also have a greater replication index and consequently be able to liberate cells with an infective capacity similar to that of other fish pathogens (Ortega et al. 2011). Therefore, the culture of the bacteria in broth medium does not affect the infective properties under *in vitro* conditions, but challenge experiments with fish are necessary to confirm that the Chilean isolates do cause the observed disease and fulfill Koch's postulates. Further studies are being done to confirm this hypothesis.

In conclusion, from a microbiological point of view, one of the major constraints on studies of *Piscirickettsia salmonis* is the ability to isolate different structural components (i.e. DNA, LPS and proteins) free of the host cell. We report for the first time the purification of DNA, LPS and membrane protein obtained from *P. salmonis* grown in a liquid medium, providing a suitable platform to simplify the preparation of *P. salmonis* cells for genetic and serological studies. Moreover, the results of the CPE test showed that *P. salmonis* grown in AUSTRAL-SRS broth maintain virulence and proteome properties, making this medium an excellent candidate for successful *P. salmonis* growth and a first-rate material for the development of low-cost vaccines as well as for monitoring antibiotic resistance.

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LITERATURE CITED

- Almendras FE, Fuentealba IC (1997) Salmonid rickettsial septicemia caused by *Piscirickettsia salmonis*: a review. *Dis Aquat Org* 29:137–144
- Arkush KD, McBride AM, Mendonca HL, Okihiro MS and others (2005) Genetic characterization and experimental pathogenesis of *Piscirickettsia salmonis* isolated from white seabass *Atractoscion nobilis*. *Dis Aquat Org* 63: 139–149
- Barnes MN, Landolt ML, Powell DB, Winton JR (1998) Purification of *Piscirickettsia salmonis* and partial characterization of antigens. *Dis Aquat Org* 33:33–41
- Birkbeck TH, Griffen AA, Reid HI, Laidler LA, Wadsworth S (2004) Growth of *Piscirickettsia salmonis* to high titers in insect tissue culture cells. *Infect Immun* 72:3693–3694
- Branson EJ, Diaz-Muñoz DN (1991) Description of a new disease condition occurring in farmed coho salmon, *Oncorhynchus kisutch* (Walbaum), in South America. *J Fish Dis* 14:147–156
- Bravo S, Campos M (1989) Coho salmon syndrome in Chile. *Am Fish Soc Fish Health Sect Newsl* 17:3
- Brocklebank JR, Speare DJ, Armstrong RD, Evelyn T (1992) British Columbia septicemia suspected to be caused by a rickettsia-like agent in farmed Atlantic salmon. *Can Vet J* 33:407–408
- Cusack RR, Groman DB, Jones SRM (2002) Rickettsial infection in farmed Atlantic salmon in eastern Canada. *Can Vet J* 43:435–440
- Cvitanich JD, Garate O, Smith CE (1991) The isolation of a rickettsia-like organism causing disease and mortality in Chilean salmonids and its confirmation by Koch's postulate. *J Fish Dis* 14:121–145
- Dannevig BH, Brudeseth BE, Gjoen T, Rode M, Wergeland HI, Evensen Ø (1997) Characterisation of a long-term cell line (SHK-1) developed from the head kidney of Atlantic salmon (*Salmo salar*). *Fish Shellfish Immunol* 7:213–226
- Finlay BB, Falkow S (1997) Common themes in microbial pathogenicity revisited. *Microbiol Mol Biol Rev* 61: 136–169
- Fryer JL, Hedrick RP (2003) *Piscirickettsia salmonis*: a Gram-negative intracellular bacterial pathogen of fish. *J Fish Dis* 26:251–262
- Fryer JL, Lannan CN, Garcés LH, Larenas JJ, Smith PA (1990) Isolation of a rickettsiales-like organism from diseased coho salmon (*Oncorhynchus kisutch*) in Chile. *Fish Pathol* 25:107–114
- Garcés LH, Larenas JJ, Smith PA, Sandino S, Lannan CN, Fryer JL (1991) Infectivity of a rickettsia isolated from coho salmon *Oncorhynchus kisutch*. *Dis Aquat Org* 11: 93–97
- Grant AN, Brown AG, Cox DI, Birkbeck TH, Griffen AA (1996) Rickettsia-like organism in farmed salmon. *Vet Rec* 138:423
- Hitchcock PJ, Brown TM (1983) Morphological heterogeneity among *Salmonella* lipopolysaccharide chemotypes in silver-stained polyacrylamide gels. *J Bacteriol* 154: 269–277

- Jamett A, Aguayo J, Miquel A, Muller I and others (2001) Characteristics of monoclonal antibodies against *Piscirickettsia salmonis*. J Fish Dis 24:205–215
- Karatas S, Mikalsen J, Steinum TM, Taksdal T, Bordevik M, Colquhoun DJ (2008) Real time PCR detection of *Piscirickettsia salmonis* from formalin-fixed paraffin-embedded tissues. J Fish Dis 31:747–753
- Kuzyk MA, Thorton JC, Kay WW (1996) Antigenic characterization of the salmonid pathogen *Piscirickettsia salmonis*. Infect Immun 64:5205–5210
- Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685
- Lannan C, Fryer J (1991) Recommended methods for inspection of fish for the salmonid rickettsia. Bull Eur Assoc Fish Pathol 11:135–136
- Marshall S, Heath S, Henriquez V, Orrego C (1998) Minimally invasive detection of *Piscirickettsia salmonis* in cultivated salmonids via the PCR. Appl Environ Microbiol 64:3066–3069
- Mauel MJ, Miller DL (2002) Piscirickettsiosis and piscirickettsiosis-like infections in fish: a review. Vet Microbiol 87:279–289
- Mauel MJ, Ware C, Smith PA (2008) Culture of *Piscirickettsia salmonis* on enriched blood agar. J Vet Diagn Invest 20:213–214
- McGann P, Rozak DA, Nikolich MP, Bowden RA, Lindler LE, Wolcott MJ, Lathigra R (2010) A novel brain heart infusion broth supports the study of common *Francisella tularensis* serotypes. J Microbiol Methods 80:164–171
- Mikalsen J, Skjaervik O, Wiik-Nielsen J, Wasmuth MA, Colquhoun DJ (2008) Agar culture of *Piscirickettsia salmonis*, a serious pathogen of farmed salmonid and marine fish. FEMS Microbiol Lett 278:43–47
- Nobbs AH, Lamont RJ, Jenkinson HF (2009) *Streptococcus* adherence and colonization. Microbiol Mol Biol Rev 73: 407–450
- OIE (World Organisation for Animal Health) (2006) Manual of diagnostic tests for aquatic animals 2006. World Organisation for Animal Health, Paris
- Olavarria VH, Gallardo L, Figueroa JE, Mulero V (2010) Lipopolysaccharide primes the respiratory burst of Atlantic salmon SHK-1 cells through protein kinase C-mediated phosphorylation of p47phox. Dev Comp Immunol 34:1242–1253
- Olsen AB, Melby HP, Speilberg 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: 35–48
- Ortega C, Rodríguez S, de Las Heras AI, Romero A, Monrás M, Enríquez R (2011) Evaluation of the level of Mx3 protein synthesis induced by infectious pancreatic necrosis virus (IPNV) strains of different infectivity. Vet Immunol Immunopathol 141:190–200
- Pot B, Vandamme P, Kersters K (1994). Analysis of electrophoretic whole-organism protein fingerprints. In: Goodfellow M, O'Donnell AG (eds) Chemical methods in prokaryotic systematics. Wiley, Chichester, p 493–521
- Rodger HD, Drinan EM (1993) Observation of a rickettsia-like organism in Atlantic salmon *Salmo salar* L., in Ireland. J Fish Dis 16:361–369
- Sokal R, Rohlf J (1980) Introducción a la bioestadística. De Reverte S.A., Barcelona
- Tsai CM, Frasch CE (1982) A sensitive silver stain for detecting lipopolysaccharides in polyacrylamide gels. Anal Biochem 119:115–119

Appendix 1. AUSTRAL-SRS broth is a supplemented media for *Piscirickettsia salmonis* culture based on peptone and yeast extract as a source of nitrogen, vitamins and minerals. The high salt content helps to simulate seawater; numerous minerals are also included to duplicate the major mineral composition of seawater. Preparation for 1 l: Dissolve ingredients 1 to 21 in 900 ml of distilled water and sterilize by autoclaving at 121°C for 15 min. Allow media to cool at room temperature and aseptically add ingredients 22 to 50. All ingredients except 50 are in mg l⁻¹. FBS: fetal bovine serum

Component	Amount (mg l ⁻¹)
Inorganic salts	
1 Ammonium nitrate	0.2
2 Boric acid	2.8
3 Calcium chloride (CaCl ₂) (anhydride)	246.1
4 Disodium phosphate	1.0
5 Ferric citrate	12.6
6 Feric nitrate	5.0
7 Magnesium chloride	741.2
8 Magnesium sulfate (MgSO ₄) (anhydride)	416.8
9 Potassium bromide	10.1
10 Potassium chloride (KCl)	109.1
11 Sodium bicarbonate (NaHCO ₃)	240.1
12 Sodium chloride (NaCl)	7123.5
13 Sodium fluoride	0.3
14 Sodium phosphate monobasic (NaH ₂ PO ₄ -H ₂ O)	14.0
15 Sodium silicate	0.5
16 Sodium sulfite	200.0
17 Strontium chloride	4.3
Other components	
18 D-glucose (dextrose)	15600.0
19 Proteose peptone no. 3	8128.1
20 Pancreatic digest of casein	7500.0
21 Soy peptone	5000.0
Amino acids	
22 Glycine	0.8
23 L-alanine	0.9
24 L-arginine	12.6
25 L-asparagine-H ₂ O	1.3
26 L-aspartic acid	1.3
27 L-cystine 2 HCl	1703.1
28 L-glutamic acid	1.5
29 L-histidine hydrochloride-H ₂ O	4.2
30 L-isoleucine	5.2
31 L-leucine	5.2
32 L-lysine	7.3
33 L-methionine	1.5
34 L-phenylalanine	3.2
35 L-proline	1.2
36 L-serine	1.1
37 L-threonine	4.8
38 L-tryptophan	1.0
39 L-tyrosine disodium salt dihydrate	5.2
40 L-valine	4.6
Vitamins	
41 Choline chloride	0.1
42 D-calcium pantothenate	0.1
43 Folic acid	0.1
44 Niacinamide	0.1
45 Pyridoxal hydrochloride	0.1
46 Riboflavin	0.0
47 Thiamine hydrochloride	0.1
48 i-inositol	0.2
49 Yeast extract	325.6
50 FBS cell culture Gibco®	100 ml