Journal of Fish Diseases 2004, **27,** 431–433

Short communication

DNA hybridization assays for the detection of *Piscirickettsia salmonis* in salmonid fish

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Keywords: diagnosis, dot-blot hybridization, *in situ* hybridization, *Piscirickettsia salmonis*, salmonids.

Since it was first recorded in 1989, *Piscirickettsia salmonis* has been a constraint on the Chilean salmon farming industry (Bravo & Campos 1989; Fryer, Lannan, Garcés, Larenas & Smith 1990). This obligate, intracellular, Gram-negative bacterium and the disease it causes, known as 'salmon rickettsial septicaemia' (Cvitanich, Gárate & Smith 1991) or 'piscirickettsiosis' (Fryer, Lannan, Giovannoni & Wood 1992), causes major losses in salmon farming in Chile (Smith, Pizarro, Ojeda, Contreras, Oyanedel & Larenas 1999).

Laboratory diagnosis of *P. salmonis* is based largely on a presumptive detection using Giemsa stained smears or on pathogen isolation by cell culture (Fryer *et al.* 1990). Confirmative diagnosis is by indirect fluorescent antibody test (Lannan, Ewing & Fryer 1991; OIE 2000), immunohistochemistry (Alday-Sanz, Rodger, Turnbull, Adams & Richards 1994; OIE 2000), and/or polymerase chain reaction (PCR) (Mauel, Giovannoni & Fryer 1996; OIE 2000; House & Fryer 2002). The interest in the use of DNA-based techniques for the diagnosis/detection of fish and shellfish pathogens, particularly those that are difficult to culture, has been steadily increasing. The present communication reports on two of these approaches, dot-blot hybridization (DBH) and *in situ* hybridization (ISH).

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Two sets of primers (PS2S-PS2AS and PS2S-PS3AS) were used as specific DNA probes against *P. salmonis* according to information reported by Mauel *et al.* (1996). Unless otherwise indicated procedures were performed at room temperature. Appropriate non-infected samples were included as controls for both DBH and ISH.

For hybridization detection, the primers were directly labelled with 11-digoxigenin-dNTPs (Roche Molecular Biochemicals, Mannheim, Germany) by PCR amplification. For the DBH assay, a phenolic extraction of nucleic acids was made from samples of kidney, liver and spleen of *P. salmonis* (SLGO-95 strain)-infected rainbow trout, *Oncorhynchus mykiss* (Walbaum), and from the supernatant of infected CHSE-214 cells. The tissue samples were obtained from an infectivity trial performed in our laboratory. Briefly, the samples were homogenized and digested with a lysis buffer (280 mM Tris, 80 mM EDTA, 45 mM SDS, 685 mM NaCl, 1.3% β-mercaptoethanol) and Proteinase K (0.2 mg mL^{-1}; GibcoBRL, Carlsbad, CA, USA), incubated at 50 °C for 1 h and subjected to a phenol/chloroform extraction. Extracted DNAs were denatured at 95 °C for 5 min, quenched on ice for 3 min and then treated with 2x SSC (standard saline citrate 20x: 300 mM NaCl, 30 mM Na citrate, pH 7.0) and blotted onto positively charged nylon membranes (Amersham Pharmacia Biotech, Piscataway, NJ, USA). The membranes and nucleic acids were cross-linked by UV radiation for 3 min and treated with a

prehybridization solution [50% formamide, 5x SSC, 0.1% *n*-laurylsarcosine, 0.02% SDS, 1% blocking reagent (Roche Molecular Biochemicals)] for 30 min at 45 °C, according to the melting points and optimal hybridization temperature. After the prehybridization the membranes were immersed in a hybridization solution with the selected set of labelled probes, which were previously denatured at 95 °C for 5 min, and incubated in a waterbath overnight at 45 °C. The hybridization solution contained the same components as the prehybridization solution except that the probes were added at 1:500 and 1:1000 (v/v). Stringency washes were performed on the membrane using buffer 1 (100 mM maleic acid, 150 mM NaCl, pH 7.5) twice for 1 min and buffer 2 (buffer 1, 1% blocking reagent, pH 7.5) once for 30 min. These washes were carried out to remove unbound probe molecules. The membranes were incubated for 30 min with anti-digoxigenin alkaline phosphatase-conjugated sheep antibody (Roche Molecular Biochemicals) at a concentration of 1:500 and 1:1000. These antibodies were diluted with buffer 2. The membranes were subsequently rinsed twice with buffer 1 for 15 min and then equilibrated with buffer 3 (100 mM Tris–HCl, pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$) for 2 min. The substrate was prepared during the equilibration step by adding 4.4 and 3.3 μl of nitroblue tetrazolium (NBT) at 75 mg mL^{-1} and 5-bromo-chloro-3-indoyl phosphate (BCIP) at 50 mg mL^{-1} (GibcoBRL), respectively, to 1 mL of buffer 3. The membranes were incubated with the substrate solution for 30 min in a covered chamber on a low speed shaker and observed for colour development every 5 min.

Formalin-fixed kidney, liver and spleen samples from *P. salmonis*-infected *O. mykiss* were embedded in paraffin and cut to obtain 5 μm sections for the ISH assay. Sections were mounted on slides treated with 3-aminopropyltriethoxysilane (Fluka, Buchs SG, Switzerland). They were dewaxed in xylene, dehydrated in ethanol and then digested with Proteinase K (100 μg mL^{-1} to 10 mg mL^{-1}) for 15 min at 37 °C. Proteinase K was diluted in buffer TE (50 mM Tris–HCl, 1 mM EDTA, pH 7.4). After digestion, sections were post-fixed in 4% formalin for 5 min and washed in 2x SSC once. At this stage, they were prehybridized with the same solution and conditions of the DBH assay, except for the use of a humid chamber where the slides were kept to prevent the solution from drying out. The hybridization process followed the same conditions as for the prehybridization except that the digoxigenin-labelled probe was added at concentrations of 1:500, 1:1000 and 1:2500. After adding the hybridization solution (500 μL), preparations were denatured at 95 °C for 5 min and then quenched on ice for 3 min. Samples were incubated in a humid chamber at 45 °C overnight. Incubation was followed by a stringency wash consisting of the application of 2x SSC, 1x SSC and 0.5x SSC solutions at 37 °C for 5 min, each step being carried out twice. The tissues were washed in buffer 1, blocked in buffer 2, and incubated for 30 min with anti-digoxigenin alkaline phosphatase-conjugated sheep antibody (Roche Molecular Biochemicals) at a concentration of 1:500 and 1:1000. For colour development the sections were first equilibrated in buffer 3 for 5 min and then exposed to the substrate solution as described for DBH. A final step for ISH was a counterstain with 0.5% Bismarck brown for 5 min, followed by dehydration and mounting with a permanent xylene medium (Entellan neu, Merck, Germany).

No positive signals were observed by DBH and ISH from non-infected samples. The bacterium was detected by DBH from *P. salmonis* infected samples from both fish tissues and cell culture supernatants. This method provided a preliminary step to check the specificity of the labelled probes, which were subsequently used on tissue sections in the ISH assay. The DBH method can easily be implemented and provides a fast and highly specific technique for the detection of *P. salmonis*.

Positive signals were observed by ISH in sections of infected kidney (Figs 1 & 2), liver and spleen. Positive signals were easily distinguished from

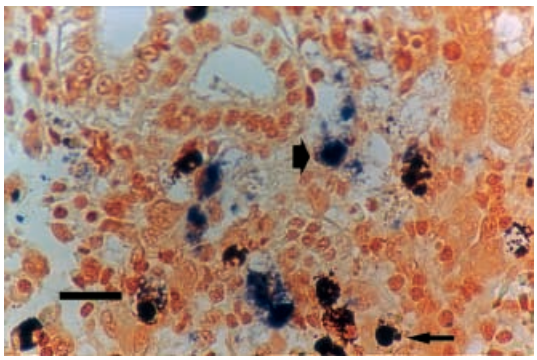

Figure 1 *In situ* hybridization. Rainbow trout kidney infected with *Piscirickettsia salmonis*. Positive signal (blue colour) is indicated by a wide arrow. A melanin granule is indicated by a narrow arrow (bar = 20 μm).

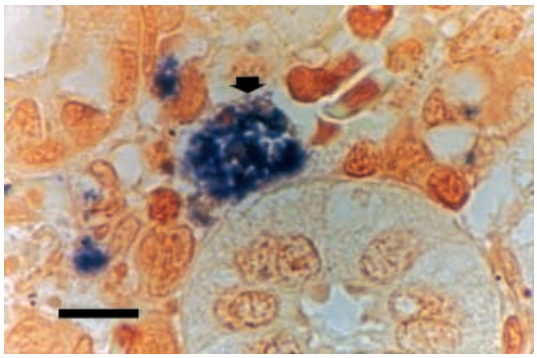

Figure 2 *In situ* hybridization. Rainbow trout kidney infected with *Piscirickettsia salmonis*. Positive intracellular signal (blue colour) is indicated by the arrow (bar = 10 μm).

melanin granules, which are frequently present in kidney and spleen. No differences were found in the results (by ISH and DBH) using either the digoxigenin-labelled PS2S-PS2AS or PS2S-PS3AS. This concurs with Mauel *et al.* (1996) who indicated that the SLGO strain of *P. salmonis* can be detected using either set of primers.

Penetration of tissue by the probes during the ISH method was accomplished using Proteinase K (Nuovo 1994). After testing different dilutions at 37 °C for 15 min it was found that the optimum concentration of Proteinase K was 100 μg mL^{-1}. Under these conditions there were stronger hybridization signals compared with the untreated sections or those treated with lower and higher concentrations of the enzyme.

Strong signals were observed either in DBH using a 1:1000 dilution of the labelled probe or a 1:500 dilution in the case of ISH. The optimum dilution of the anti-digoxigenin alkaline phosphatase conjugated antibody was 1:500 for ISH and 1:1000 for DBH at an incubation temperature of 37 °C for 30 min. The binding of the BCIP-NBT substrate for 1 h for ISH and 15 min for DBH gave a strong signal and minimal background. Incubating the substrate beyond the times given here did not significantly increase the signal, but increased non-specific staining.

The DNA-hybridization approaches reported in this communication provide a valuable tool for detection of *P. salmonis* affecting salmonid fish.

Acknowledgement

This work was supported by Grant 1010544 of the Chilean Fund for Science and Technology (FONDECYT).

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Received: 4 September 2003
Revision received: 19 April 2004
Accepted: 21 April 2004

