Development of polymerase chain reaction assays for detection, identification, and differentiation of *Piscirickettsia salmonis*

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ABSTRACT: A nested polymerase chain reaction (PCR) was developed to detect genomic DNA of *Piscirickettsia salmonis*, the causative agent of an epizootic disease in salmonids. The nested PCR assay, which used general bacterial 16S rDNA primers in the first amplification reaction, and *P. salmonis*-specific primers in a second reaction, allowed detection of less than 1 *P. salmonis* tissue culture infectious dose 50 ($TCID_{50}$). Using the *P. salmonis*-specific primers in a single PCR reaction allowed the detection of 60 $TCID_{50}$. The specificity of the PCR was assessed with a panel of 4 salmonid and 15 bacterial genomic DNA preparations. Amplification products were produced only with *P. salmonis* DNA. Restriction fragment length polymorphism (RFLP) analysis of the complete 16S gene PCR products demonstrated that 1 isolate, EM-90, was unique. Two additional primers were developed and used in PCR assays that differentiated EM-90 from the 4 other *P. salmonis* isolates tested.

KEY WORDS: *Piscirickettsia salmonis* · DNA · PCR · Fish disease · Salmonid · Rickettseae

INTRODUCTION

Salmonid mariculture in Chile has undergone rapid growth and development during the last 2 decades. In the late 1980s a number of mariculture facilities began experiencing extensive losses due to an infectious disease of unknown etiology. Signs of the disease became evident 6 to 8 wk after fish were moved to salt water netpens. In 1989 mortality up to 90% was reported in some coho salmon *Oncorhynchus kisutch* facilities in the Puerto Montt area of southern Chile (Bravo & Campos 1989). The etiologic bacterium of this disease was isolated (Fryer et al. 1990) and characterized (Fryer et al. 1992). The causative agent was designated *Piscirickettsia salmonis* (gen. nova, sp. nova), an intracellular parasite and the first such rickettsial-like agent isolated from fish. The disease was designated piscirickettsiosis. Since *P. salmonis* first appearance in Chile, *Piscirickettsia* or *Piscirickettsia*-like organisms

have been reported from salmonids in Canada (Evelyn 1992), Norway (Olsen et al. 1993) and Ireland (Rodger & Drinan 1993).

Presumptive diagnoses of piscirickettsiosis are made by observing gross signs of disease, histological staining of smears or tissue sections, electron microscopy, isolation in fish cell cultures and consideration of the previous disease history in the rearing facility. Currently, confirmation of *Piscirickettsia salmonis* is accomplished by indirect fluorescent antibody test (IFAT; Lannan et al. 1991). While the IFAT is invaluable, it uses polyclonal antibodies that do not differentiate between isolates of *P. salmonis*. Electron microscopy is time consuming and expensive, and organisms of similar morphology are difficult to differentiate. *P. salmonis* is sensitive to many of the antibiotics commonly used in cell culture media, making isolation of the agent from tissues of infected fish difficult and time consuming due to contamination. The problems associated with the above diagnostic methods demonstrate the need for a highly specific, sensitive and rapid assay for diagnosis of piscirickettsiosis.

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In addition to the isolates from salmonids, rickettsia-like organisms have recently been isolated or reported from several species of tilapia (*Oreochromis* and *Tilapia* sp.) in Taiwan (Chern & Chao 1994) and Japan (Wada et al. 1995). Other rickettsia-like organisms have been reported in the blue-eyed plecostomus *Panaque suttoni* from Colombia, South America (Khoo et al. 1995) and juvenile sea-bass *Dicentrarchus labrax* in France (Comps 1996). The discovery of these agents demonstrates the need for a highly specific, sensitive assay which can differentiate *Piscirickettsia salmonis* from other rickettsia-like agents.

This report describes the development of a nested polymerase chain reaction (PCR) for detecting *Piscirickettsia salmonis* genomic DNA from cultured fish cells and the tissues of infected fish. This assay proved to be both specific and sensitive. Two additional PCR primers were developed that differentiated between *P. salmonis* isolate EM-90 and the other 4 isolates of *P. salmonis* examined in this study.

MATERIAL AND METHODS

Bacterial strains and culture. The species examined, their strain designations and isolation sources are listed in Table 1. The bacterium *Neorickettsia helminthoeca* was obtained along with the metacercariae of its host *Nanophyetus salmincola* from kidney tissue of adult steelhead trout *Oncorhynchus mykiss* (Gebhard et al. 1966). No attempt was made to isolate the bacterium from the metacercariae.

Piscirickettsia isolates were grown at 15°C in chinook salmon embryo cell line (CHSE 214, Lannan et al. 1984) in antibiotic-free Eagle's minimum essential medium (MEM) with Earle's salts (Sigma Chemical Co., St. Louis, MO, USA) supplemented with 10% fetal bovine serum (Hyclone Laboratories, Inc., Logan, UT, USA) until lysis of the cell sheet was near completion (approximately 14 d).

DNA isolation. *Piscirickettsia salmonis* DNA was isolated using DNA-STAT 60 (Tel-Tex, Inc., Friendswood, TX, USA) following the manufacturer's protocol. Briefly, 7 ml of DNA-STAT 60 was added to 25 cm² cultures near complete lysis and the cells and supernatant passed through a pipette 5 times to disrupt the remaining intact CHSE 214 cells and lyse the bacterial cells. Next, 1.5 ml of chloroform was added, the mixture was shaken for 30 s and then centrifuged at $16\,000 \times g$ for 15 min at 4°C in a microcentrifuge. The upper layer was transferred to a second tube and DNA precipitated with 500 µl of 100% isopropanol. After 10 min at room temperature the DNA was pelleted by centrifugation at $16\,000 \times g$ at 4°C for 10 min in a microcentrifuge. The supernatant was then removed, the pellet vacuum dried for 10 min and resuspended in 200 µl TE (100 mM Tris pH 8.0, 10 mM EDTA).

The Norwegian isolate, NOR-92, was contaminated with a mycoplasma. To overcome this, amplification was performed on a plasmid (pCRII®, Invitrogen, San Diego, CA, USA) that contained the NOR-92 16S gene as an insert. Due to limited DNA availability, isolates LF-89 and EM-90 were also amplified from plasmid inserts.

Table 1. Bacteria examined in this study

Species[a]	Strain designation	Original isolation source
Piscirickettsia salmonis	LF-89 ATCC VR 1361	Coho salmon *Oncorhynchus kisutch*
	EM-90	Atlantic salmon *Salmo salar*
	SLGO-94	Rainbow trout *Oncorhynchus mykiss*
	ATL-4-91	Atlantic salmon *S. salar*
	NOR-92	Atlantic salmon *S. salar*
Vibrio anguillarum type 1	LS174	Chinook salmon *Oncorhynchus tshawytscha*
Pseudomonas fluorescens	4-13-78	Unknown
Edwardsiella tarda	ATCC 15948	Human
Edwardsiella ictaluri	ATCC 33202	Channel catfish *Ictalurus punctatus*
Pasteurella piscicida	7-1-77	Unknown
Carnobacterium piscicola	LBK1-70	Rainbow trout *O. mykiss*
Lactococcus lactis	ATCC 19435	Unknown
Aeromonas hydrophila	ATCC 14715	Coho salmon *O. kisutch*
Aeromonas salmonicida	ATCC 33685	Atlantic salmon *S. salar*
Yersinia ruckerii	HI-70	Rainbow trout *O. mykiss*
Staphylococcus aureus	ATCC 35548	Unknown
Renibacterium salmoninarum	D6	Coho salmon *O. kisutch*
Flexibacter psychrophilus	SR1-77	Coho salmon *O. kisutch*
Escherichia coli	ATCC 33430	Human
Lactococcus piscicum	7-77	Coho salmon *O. kisutch*

[a] All non *P. salmonis* species were provided by the Oregon State University Department of Microbiology Fish Pathogen Culture Collection

Chromosomal DNA from each of the non-*Piscirickettsia salmonis* bacterial strains was prepared as above using DNA-STAT 60 with the following modifications: 5 ml Lennix L broth cultures were incubated, with shaking, at either 16 or 37°C; the bacterial cells were pelleted and resuspended in 1.5 ml DNA-STAT 60; 300 µl of chloroform was added. DNA from rainbow trout *Oncorhynchus mykiss*, chinook *Oncorhynchus tshawytscha*, coho, and Atlantic salmon *Salmo salar* were provided by J. Leong (Dept. of Microbiology, Oregon State University, Corvallis, OR, USA). As a confirmation of the quality of the bacterial DNA, 16S genes were amplified prior to performing the nested PCR (data not shown).

Infected coho salmon kidney and spleen tissues fixed in ethanol were provided by P. Smith (Veterinary Sciences, University of Chile, Santiago, Chile) from a culture facility in Chile that was experiencing an epizootic of piscirickettsiosis. *Piscirickettsia salmonis* infection was confirmed by observation and histological staining for the presence of rickettsia-like organisms in host cells. Healthy coho salmon tissues obtained from the Oregon Department of Fish and Wildlife, Fall Creek Hatchery, Alsea, OR, USA, served as negative controls, since there is no history of *P. salmonis* in Oregon.

Fish infected with *Nanophyetus salmincola*, the host of *Neorickettsia helminthoeca*, were obtained from a location where the salmon poisoning syndrome is endemic (Gebhard et al. 1966). The genomic DNA of *N. helminthoeca*, its host *N. salmincola* and the *N. salmincola* fish host, steelhead trout, were isolated together from kidney tissue using DNA-STAT 60 as previously described. DNA was also isolated from infected tissue by concentration of the *N. salmincola* metacercariae as follows: 1 g of infected steelhead kidney tissue was homogenized in 50 ml phosphate-buffered saline (PBS), the metacercariae were allowed to settle for 4 h (Gebhard et al. 1966), the supernatant removed and the DNA extracted with DNA-STAT 60. The number of metacercariae per gram of host tissue was determined by counting in a haemocytometer. Staining with Giemsa was used to establish the presence of rickettsia-like bodies in the metacercaria.

Salmonid kidney or spleen tissues (100 mg), negative control or *Piscirickettsia salmonis* infected, were digested for 3 h at 65°C in 250 µl of lysis buffer (50 mM KCl, 10 mM Tris pH 7.8, 2.5 mM MgCl$_2$, 0.1% gelatin, 0.45% NP40, 0.45% Tween 20 with 1 mg ml^{-1} proteinase K added just before use), followed by boiling for 10 min, quenching on ice and centrifugation to remove cell debris. Of the supernatant, 5 µl was used for PCR.

Oligonucleotide design and synthesis. The *Piscirickettsia*-specific primers (Table 2) were designed by comparing the published sequence (Fryer et al. 1992, EMBL accession no. X60783) and 20 other published bacterial sequences using the Genetic Data Environment (GDE version 2.2) software package. The proposed primers were further tested for specificity using a commercially available software package (Amplify; University of Wisconsin, Madison, WI, USA) and the Ribosomal Data Base project program Check Probe (Maidak et al. 1994). From 16S rDNA sequence data of 5 *P. salmonis* isolates (GenBank accession numbers: LF-89 U36941, EM-90 U36940, ATL-4-91 U36915, NOR-92 U36942, SLGO-94 U55015) 2 additional primers, PS3AS and EM90AS, were developed (Table 2). The design of the universal primers EubA and EubB has been described (Giovannoni 1991). The oligonucleotides were synthesized at the Oregon State University Center for Gene Research and Biotechnology Central Services facility.

PCR amplification and Restriction Fragment Length Polymorphism (RFLP). In the first amplification of the nested PCR, 5 µl of DNA lysate was added to 45 µl of reaction mixture consisting of 1 × PCR buffer (Promega, Madison, WI, USA; 10 mM Tris HCl (pH 9.0), 1.5 mM MgCl$_2$, 50 mM KCl, 0.1% Triton × 100), 200 µM each of dATP, dCTP, dTTP and dGTP, 1 µM EubA primer, 1 µM EubB primer and 2.5 U Taq DNA polymerase (Promega), and covered with 50 µl of mineral oil. The mixture was denatured at 94°C for 2 min and amplification was performed with 35 cycles of 94°C for 1 min, 50°C for 2 min, and 72°C for 3 min. The tubes were then held at 4°C.

The second amplification was performed by adding 3 µl of the first PCR products to the reaction mixture containing 2 µM each of PS2S and PS2AS primers in-

Table 2. Sequences and specificity of primers utilized for the identification or differentiation of *Piscirickettsia salmonis* strains

Primer (Location[a])	Sequence (5'-3')	Specificity
EubB (27F)	AGAGTTTGATCMTGGCTCAG	Eubacterial
PS2S (223F)	CTAGGAGATGAGCCCGCGTTG	*P. salmonis* (all strains)
PS2A2 (690R)	GCTACACCTGCGAAACCACTT	*P. salmonis* (all strains)
PS3AS (1032R)	TCCCGAAGGCACTTCCGCATCTC	*P. salmonis* (LF-89 type strain)
EM90AS (1032R)	TCCCGAAGGCACATCAATATCTCTATC	*P. salmonis* (EM-90)
EubA (1518R)	AAGGAGGTGATCCANCCRCA	Eubacterial

[a]F: forward; R: reverse; numbering corresponds to *Escherichia coli* 16S gene

stead of the EubA and EubB primers. Reaction conditions were: 35 cycles of 94°C for 1 min, 61°C for 2 min, and 72°C for 3 min. Alternatively, amplifications with the primer sets PS2S-PS3AS or PS2S-EM90AS were performed by adding 2 μM of each primer to the above reaction mixture. The reaction conditions were modified as follows: 35 cycles of 94°C for 1 min, 65°C for 2 min, and 72°C for 3 min. Amplifications from plasmid DNA were achieved with the same reaction conditions as the genomic DNA. Aliquots (10 μl) of the PCR reaction mixture were electrophoresed in 2% agarose 1 × TAE (40 mM Tris acetate/1 mM EDTA) gel containing 1 mg per 50 ml ethidium bromide and photographed under UV transillumination. Aliquots (10 μl) of the PCR reaction products were digested with *EcoRI* or *PstI* (Promega) following the manufacturers instructions and visualized as described.

PCR sensitivity. To define the sensitivity of the PCR assay *Piscirickettsia salmonis* DNA was serially diluted and 6×10^7 tissue culture infectious dose 50 ($TCID_{50}$) to 0.6 $TCID_{50}$ of DNA was added to the reaction mixture. The *P. salmonis* cells were stained with Giemsa and counted with a haemocytometer for comparison with the $TCID^{50}$ counts.

RESULTS

Development of the PCR

Amplification of *Piscirickettsia salmonis* genomic DNA with primers PS2S and PS2AS resulted in a product with the predicted 476 bp length (Fig. 1A). Verification that the product was from the 16S gene of *P. salmonis* was determined by sequencing the LF-89 PCR product and aligning it to the *P. salmonis* LF-89 sequence (Fryer et al 1992, EMBL accession no. X60783). Amplification using PS2S and either PS3AS or EM90AS resulted in the appearance of the predicted 816 bp PCR products (Fig. 2).

Detection threshold of the PCR

Amplification of 10-fold serial dilution's of *Piscirickettsia salmonis* DNA indicated that less than 1 $TCID_{50}$ could be detected in a 2% agarose gel with the nested PCR (Fig. 3A). However, for reasons that remain unclear, amplification of the 467-bp product from 6×10^4 $TCID_{50}$ was consistently less than that obtained from 6×10^3 $TCID_{50}$ or less (Fig. 3A). When using DNA isolated from infected fish cell cultures or purified *P. salmonis* (Lannan et al. 1991), a single amplification step with the PS2S and PS2AS primers was sufficient to detect the *P. salmonis* 16S gene at 60 $TCID_{50}$ (Fig. 3B).
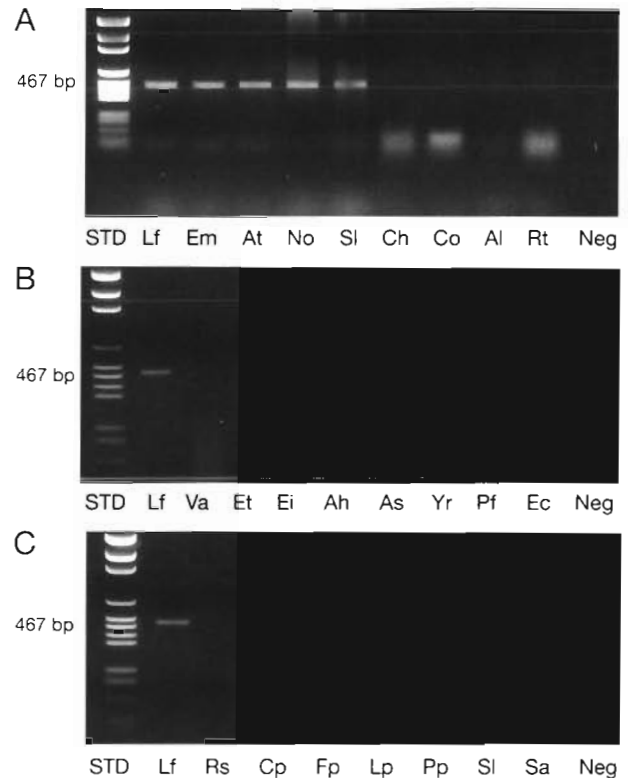

Fig. 1 Specificity of *Piscirickettsia salmonis* PCR using DNA from various bacterial and salmonid DNAs. STD = pGEM DNA standard (Promega). Neg = no DNA negative control. Lf = *P. salmonis* isolate LF-89, used as a positive control in gels B and C. (A) *P. salmonis* isolates: Lf = LF-89, Em = EM-90, At = ATL-4-91, No = NOR-92, Sl = SLGO-94, Ch = chinook salmon *Oncorhynchus tshawytscha*, Co = coho salmon *Oncorhynchus kisutch*, Al = Atlantic salmon *Salmo salar*, Rt = rainbow trout *Oncorhynchus mykiss*. (B) Va = *Vibrio anguillarum* type 1, Et = *Edwardsiella tarda*, Ei = *Edwardsiella ichtaluri*, Ah = *Aeromonas hydrophila*, As = *Aeromonas salmonicida*, Yr = *Yersinia ruckerii*, Pf = *Pseudomonas fluorescens*, Ec = *Escherichia coli*. (C) Rs = *Renibacterium salmoninarum*, Cp = *Carnobacterium piscicola*, Fp = *Flexibacter psychrophilus*, Lp = *Lactococcus piscium*, Pp = *Pasteurella piscicida*, Ll = *Lactococcus lactis*, Sa = *Staphylococcus aureus*

Fig. 2. Differentiation of *Piscirickettsia salmonis* isolates by PCR. Both assays produce an 816 bp product. Isolates: Lf = LF-89, Em = EM-90, At = ATL-4-91, No = NOR-92, Sl = SLGO-94. PS3AS indicates amplification using the primer pair PS2S-PS3AS. EM90AS indicates amplification using the primer pair PS2S-EM90AS. STD = pGEM DNA markers. Neg = no DNA negative control
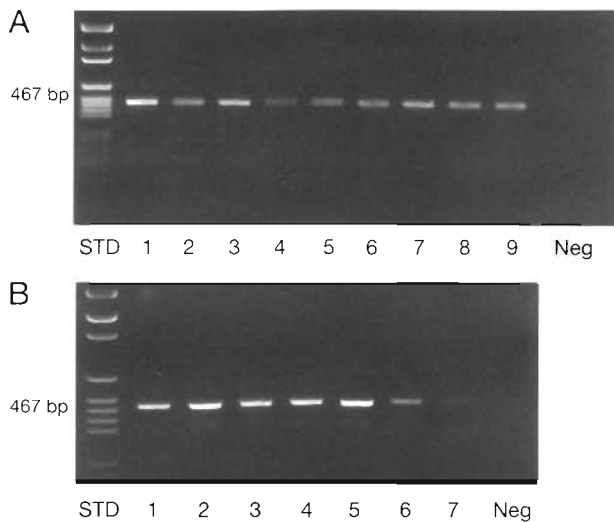
Fig. 3. Detection thresholds of the nested (A) (EubA-EubB primer pair first round, PS2S-PS2AS primer pair second round) and single round PCRs and (B) (PS2S-PS2AS primer pair) PCRs. Each lane represents the equivalent DNA, in each 50 µl PCR, from the following number of tissue culture infectious dose 50 ($TCID_{50}$): (1) 6×10^7 $TCID_{50}$, (2) 6×10^6 $TCID_{50}$, (3) 6×10^5 $TCID_{50}$, (4) 6×10^4 $TCID_{50}$, (5) 6×10^3 $TCID_{50}$, (6) 600 $TCID_{50}$, (7) 60 $TCID_{50}$, (8) 6 $TCID_{50}$, (9) 0.6 $TCID_{50}$. STD = pGEM DNA markers (Promega). Neg = no DNA negative control

Performing 2 sequential amplifications using the species-specific primers PS2S and PS2AS produced variable yield and was not consistently observed in agarose gels (data not shown).

Restriction Fragment Length Polymorphism (RFLP)

All 5 isolates produced a product of approximately 1540 bp when amplified with the universal primers EubA and EubB. When these products were cut with EcoRI LF-89, ATL-4-91, NOR-92 and SLGO-94, all produced 2 bands (994 bp and 546 bp). EM-90 was not cut by the restriction endonuclease EcoRI. The restriction endonuclease PstI produced 3 bands (541 bp, 519 bp, and 480 bp) from the 16S PCR products of isolates LF-89, ATL-4-91, NOR-92 and SLGO-94 and 2 bands (1058 bp and 482 bp) from EM-90 (Fig. 4). The use of RFLP demonstrated that EM-90 was distinguishable from the other 4 isolates of Piscirickettsia salmonis.

Specificity of the PCR

When using the primer combination of PS2S and PS2AS with the 18 bacterial and 4 salmon DNAs tested, the PCR produced a product only with the 5 isolates of Piscirickettsia salmonis (Fig. 1). PCR performed with the P. salmonis-specific primers did not produce products when Neorickettsia/Nanophyetus DNA from infected kidney tissues or concentrated metacercaria were used as templates (Fig. 5).

The nested assay distinguished between Piscirickettsia salmonis when compared with other bacteria including 12 species of fish pathogens, and no products were produced from the DNA from the 4 species of salmon examined (Fig. 1). When using PS2S and PS3AS as primers, a product was produced with all isolates of P. salmonis examined except EM-90. Amplification using PS2S and EM90AS produced a product only with isolate EM-90 (Fig. 2).

Amplification from infected tissue

With a single amplification of the infected fish tissue, using the P. salmonis-specific primers, 50% of the reactions produced the expected product (gel not shown). All tissue infected with P. salmonis revealed the expected 467 bp product when the nested PCR amplification was performed (Fig. 6). Close examination of the gel was required to observe the bands in lanes 4 and 5 and they could easily have been improperly identified as negative. The 467 bp band was not observed in any of the non-infected tissue or the preparations containing no DNA.

DISCUSSION

The PCR has shown potential for improving the diagnosis of infectious diseases caused by fastidious or slowly growing microorganisms (Eisenstein 1990). PCR assays have been used for the detection of a number of other rickettseae, i.e. Coxiella burnetii (Mallavia et al. 1990), Rickettsia rickettsii (Tzianabos et al. 1989), and Rickettsia typhi (Carl et al. 1990).
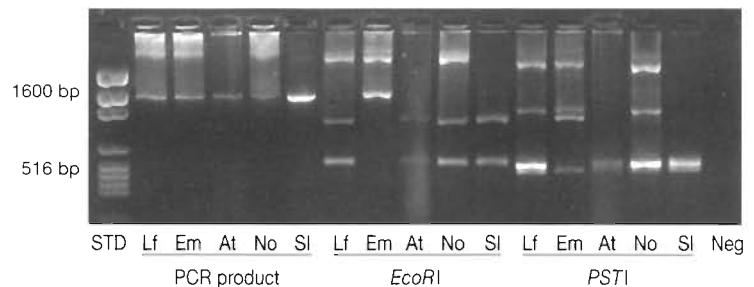

Fig. 4. PCR-RFLP patterns of 16S rDNA from Piscirickettsia salmonis isolates digested with EcoRI or PSTI. Lf = LF-89, Em = EM-90, At = ATL-4-91, No = NOR-92, Sl = SLGO-94. The 16S rDNA genes for isolates LF-89, EM-90 and NOR-92 were amplified from inserts in the pCRII plasmid. STD = pGEM DNA markers. Neg = no DNA negative control
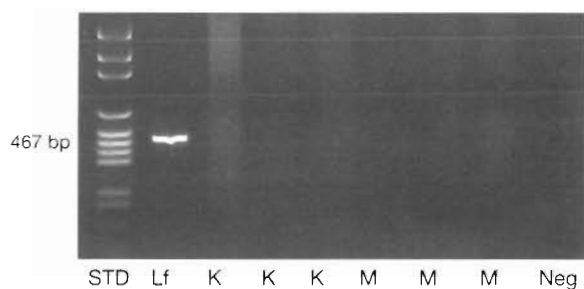
Fig. 5. Specificity of *Piscirickettsia salmonis* PCR using DNA from adult steelhead trout *Oncorhynchus mykiss* kidney tissue infected with *Nanophyetus salmonicola* metacercariae the host for *Neorickettsia helmintheoca*. Lanes marked K use infected kidney tissue. Lanes marked M use DNA from concentrated metacercaria. The metacercaria averaged 6.8×10^5 per gram of kidney tissue. STD = pGEM DNA markers (Promega). Lf = LF-89 positive control. Neg = no DNA negative control

Here we have described the development of PCR assays for the detection of *Piscirickettsia salmonis* DNA in fish cell cultures and tissues of infected fish. Amplification using the primer pair PS2S-PS2AS can distinguish *P. salmonis* from its fish host and other bacteria. The PCR assays using PS2S and either PS3AS and EM90AS distinguished between EM-90 and the other isolates of *P. salmonis*. The nested PCR was more sensitive and reliable than 2 sequential amplifications using the *P. salmonis*-specific primers.

The nested PCR approach increased the sensitivity of the assay from 60 $TCID_{50}$ to less than 1 $TCID_{50}$, which should allow detection early in the infection when the quantity of bacteria in the tissue is low. The variation in the amount of product produced with the nested PCR is due to the variation in the quantity of *Piscirickettsia salmonis* in the infected tissue. Therefore, replicate amplifications are recommended to confirm negative results. One $TCID_{50}$ may represent infection with more than 1 bacterial cell leading to an underestimate of the number of *P. salmonis* cells present. Therefore, the detection of less than 1 $TCID_{50}$ does not mean that less than 1 *P. sal-*

monis bacterium can be detected; instead it probably reflects the affinity of *P. salmonis* to host cell membranes, which leads to clumping. DNA from nonviable *P. salmonis* bacterial cells will be present in the preparation, further complicating the quantification of the number of infectious bacterial cells represented by the DNA preparation. We attempted to overcome this by measuring the $TCID_{50}$, staining the cells with Giemsa and counting by haemocytometer. The numbers agreed within 15% between the 2 methods.

Currently, preliminary diagnosis of *Piscirickettsia salmonis* relies on the presence of gross signs of disease and Giemsa staining of infected fish tissue, with confirmation by IFAT (Lannan et al. 1991). However, *P. salmonis* infections which are in the early stages when few bacterial cells are present or from fish which are asymptomatic carriers could go undetected by these tests. The sensitivity of the nested PCR assay will allow identification of the infectious agent below the detection limits of microscopic examination. Using the nested PCR will also allow the tissue distribution of the bacterium to be studied early in the infection prior to the onset of disease signs, thus resulting in a better understanding of the transmission and pathogenesis of *P. salmonis*.

The entire process of tissue preparation, DNA isolation, amplification and visualization of the PCR products can be accomplished in 1 to 2 d. The PCR/RFLP procedures take considerable less time than culturing *Piscirickettsial* agents and can differentiate between strains which cannot be distinguished by polyclonal IFAT.

A number of rickettsia-like bacteria have been recently observed in a variety fish hosts (Chern & Chao 1994, Khoo et al. 1995, Wada et al. 1995, Comps 1996). The discovery of these agents makes it likely that *Piscirickettsia salmonis* is only the first aquatic rickettsia-like bacterium to be characterized. Using the nested PCR assay described here will allow *P. salmonis* to be rapidly distinguished from the other agents and help in understanding the classification of this emerging group of microorganisms.

The PCR methods presented here are rapid, sensitive and specific tests for *Piscirickettsia salmonis* and will be useful in defining the biology, taxonomy and ecological characteristics of this agent. The sensitivity and specificity of these PCR procedures will be useful for defining the mode(s) of transmission, the natural host(s), the reservoir(s), and the geographical distribution of *P. salmonis* and other related rickettsia-like organisms.
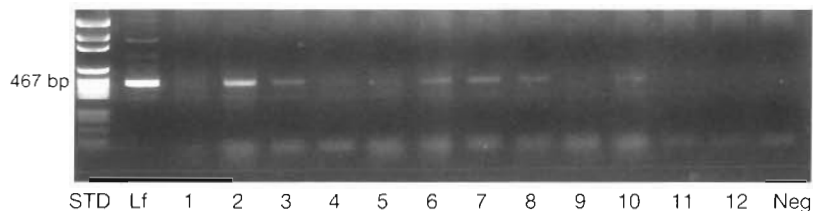

Fig. 6. Agarous gel electrophoresis of *Piscirickettsia salmonis*-specific nested PCR using infected fish tissue. Lanes 2–8, 10 were *P. salmonis* positive fish tissue samples. Lanes 1, 9, 11, 12 were *P. salmonis* negative fish tissue samples. 467 bp indicates the expected PCR product. STD = pGEM DNA markers. Neg = no DNA negative control

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