Vol. 97: 135–142, 2011
doi: 10.3354/dao02395

DISEASES OF AQUATIC ORGANISMS
Dis Aquat Org

Published December 6

Multiplex PCR for the detection of *Piscirickettsia salmonis*, *Vibrio anguillarum*, *Aeromonas salmonicida* and *Streptococcus phocae* in Chilean marine farms

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ABSTRACT: A multiplex (m-)PCR-based protocol was designed for the simultaneous detection of the main marine bacterial pathogens in Chilean salmon farms: *Streptococcus phocae*, *Aeromonas salmonicida*, *Vibrio anguillarum* and *Piscirickettsia salmonis*. Each of the 4 oligonucleotide primer pairs exclusively amplified the target gene of the specific bacterial pathogen. The detection limit of the m-PCR using purified total bacterial DNA was 50 pg µl^{-1} for *V. anguillarum*, 500 fg µl^{-1} for *P. salmonis*, and 5 pg µl^{-1} for *S. phocae* and *A. salmonicida*. This corresponded to average limits in the m-PCR sensitivity of 3.69×10^5 CFU ml^{-1} of *V. anguillarum*, 1.26×10^4 CFU ml^{-1} of *S. phocae*, and 5.33×10^4 CFU ml^{-1} of *A. salmonicida*, while the detection limits for the spiked fish tissues, regardless of the sample (spleen, kidney, liver or muscle) were $2.64 \pm 0.54 \times 10^7$ CFU g^{-1} for *V. anguillarum*, $9.03 \pm 1.84 \times 10^5$ CFU g^{-1} for *S. phocae*, $3.8 \pm 0.78 \times 10^3$ CFU mg^{-1} for *A. salmonicida* and 100 *P. salmonis* cells. However, high amounts of DNA from 3 bacterial species had a reduction of ~1 log-unit on the amplification sensitivity of *S. phocae* or *A. salmonicida* when these were present in lower concentration in the multiplex reaction. The assay described in this study is a rapid, sensitive and efficient tool to detect the presence of *S. phocae*, *A. salmonicida*, *V. anguillarum* and *P. salmonis* simultaneously from pure cultures and tissues from clinically diseased fish. Therefore, it may be a useful alternative to culture-based methods for the diagnosis of infections in fish obtained from Chilean salmon farms.

KEY WORDS: Multiplex PCR · Fish pathogens · Atlantic salmon · Analytic sensitivity test · Specificity test

INTRODUCTION

The intensive exploitation of salmonids is an activity of high economic importance in Chile, this country being second largest producer of Atlantic salmon *Salmo salar* in the world (Parada 2010). The production mainly takes place in the south of Chile and is dominated by the marine culture of Atlantic salmon with an estimated production of ~400 000 t in 2007 (www.salmonchile.cl). However, after the outbreak of infectious salmon anaemia (ISA) in June 2007 (Godoy et al. 2008), the Chilean aquaculture industry had a reduction of 30% in total production during 2009 compared to 2007.

In spite of the ISA outbreak, *Piscirickettsia salmonis*, the causative agent of piscirickettsiosis or salmonid rickettsial septicaemia, is considered the principal agent affecting and causing mortalities in the

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marine Chilean farming every year with annual economic losses of US $100 million http://aqua.merck-animal-health.com/diseases/piscirickettsiosis/productadditional_127_113333.aspx. Affected fish are dark in colouration, show inappetence, are lethargic and swim near the surface or in edges of the cages. Internally enlarged spleen, discoloured kidney and anaemia, the principal characteristic of the disease, can be observed. In addition, some fish show distinct circular nodules in the liver (see Fryer & Hedrick 2003 for review).

In the last decade, incidence of other pathogens such as *Streptococcus phocae* (Romalde et al. 2008), *Vibrio anguillarum* (Silva-Rubio et al. 2008) and atypical *Aeromonas salmonicida* has increased in Chilean salmon culture, provoking significant mortalities also in rainbow trout *Onchorhynchus mykiss* and Pacific salmon *Oncorhynchus kisutch* (Bravo 2000, Godoy et al. 2010).

Each disease is diagnosed presumptively based on the clinical signs of the affected fish. However, *Streptococcus phocae*, *Aeromonas salmonicida*, *Vibrio anguillarum* and *Piscirickettsia salmonis* often appear in mixed infections, increasing the possibility of misdiagnosis commonly attributed to *P. salmonis* perhaps due to the high incidence of the organism in net-cages. In order to prevent and control outbreaks associated with mixed infections by these 4 pathogens, development of an effective diagnostic method is an important step.

The majority of Chilean laboratories employ isolation from fish tissues on agar media followed by identification using biochemical and/or serological techniques. Until the development of an agar culture medium (Mikalsen et al. 2007, Mauel et al. 2008), cell culture in Chinook salmon embryo (CHSE) cells was considered the gold standard to confirm *Piscirickettsia salmonis* (Lannan & Fryer 1991), but both culture methods require intensive labour and are technically demanding.

Polymerase chain reaction (PCR) has been used for rapid detection and identification of each of the above microorganisms not only from plate cultures but also in clinical samples from several fish organs (i.e. kidney, gill, heart, spleen and liver) without further isolation (Gustafson et al. 1992, Miyata et al. 1995, Hirono et al. 1996, Mauel et al. 1996, 1999, Marshall et al. 1998, Gonzalez et al. 2003, Alber et al. 2004, Nilsson et al. 2006, Avendaño-Herrera 2008, Beaz-Hidalgo et al. 2008, Hassan et al. 2008).

Nevertheless, it would be a relatively costly and laborious process if single primer sets were used on a large number of samples in individual PCRs (Altinok et al. 2008). An alternative to singleplex PCR would be the use of simultaneous detection of fish pathogenic bacteria using a multiplex PCR (m-PCR) approach. In the present study, an m-PCR assay was developed for the simultaneous detection of *Streptococcus phocae*, *Aeromonas salmonicida*, *Vibrio anguillarum* and *Piscirickettsia salmonis* for the rapid and cost effective detection of these pathogens in fish from Chilean salmon aquaculture.

MATERIALS AND METHODS

Bacterial strains and growth conditions

The bacterial strains used to evaluate the m-PCR method in this study are presented in Table 1. This

Table 1. Strains used in the present study and results using the specific m-PCR detection method. AtS: Atlantic salmon; RbT: rainbow trout; CoS: Pacific salmon; ChS: Chinook salmon; Tb: turbot; ATCC: American Type Culture Collection (Rockville, USA). *Atypical and **typical strains

Bacterial strain	Source	Isolation source (AtS/RbT/CoS/ChS/Tb)	No. of strains detected
Isolated from diseased fish			
Piscirickettsia salmonis	Laboratory collection	0/3/3/1/0	0/3/3/1/0
Aeromonas salmonicida	Laboratory collection	8*/0/0/0/3**	8/0/0/0/3
Streptococcus phocae	Laboratory collection	13/0/0/0/0	13/0/0/0/0
Vibrio anguillarum	Laboratory collection	1/0/2/0/3	1/0/2/0/3
Reference strains			
Streptococcus phocae ATCC 51973^T	ATCC	*Phoca vitulina*	1
Aeromonas salmonicida subsp. *salmonicida* ATCC 33658	ATCC	*Salmo salar*	1
Aeromonas salmonicida subsp. *achromogenes* ATCC 33659	ATCC	*Salmo trutta*	1
Vibrio anguillarum ATCC 43307	ATCC	*Salmo gairdneri*	1
Piscirickettsia salmonis ATCC VR-1361	ATCC	*Oncorhynchus kisutch*	1

collection comprises 31 isolates from Atlantic salmon *Salmo salar*, Pacific salmon *Oncorhynchus kisutch*, rainbow trout *O. mykiss* and Chinook salmon *O. tshawytscha* in different Chilean salmon farms and 6 isolates from turbot *Psetta maxima*. The type strain *Streptococcus phocae* ATCC 51973^T, and the reference strains *Aeromonas salmonicida* subsp. *salmonicida* ATCC 33658, *A. salmonicida* subsp. *achromogenes* ATCC 33659, *Vibrio anguillarum* ATCC 43307 and *Piscirickettsia salmonis* ATCC VR-1361 (equivalent to LF-89) were used as positive controls and were obtained from the American Type Culture Collection.

In addition, reference strains of other pathogens from fish and mammals also obtained from outbreaks were included to test specificity of the m-PCR (Table 2). The identity of each isolate was confirmed by standard phenotypical and microscopical techniques as reported by MacFaddin (1980) and also in some cases using PCR-based analysis or serological assays.

For all experiments, *Aeromonas salmonicida* and *Vibrio anguillarum* strains were routinely grown on tryptone soya agar or broth supplemented with 1% (w/v) sodium chloride (TSA-1 or TSB-1, respectively) at 20°C for 24 to 72 h. Columbia sheep blood agar plates (CBA; AES Laboratory) were used to culture *Streptococcus phocae*. For *Piscirickettsia salmonis*, BFCG agar (5% sheep blood agar plates that had the addition of 3% fetal bovine serum [FBS], 0.1% cys-

Table 2. Bacteria from other species included in this study as negative controls in the m-PCR analysis. NBRC: National Institute of Technology and Evaluation (NITE) Biological Resource Center (Osaka, Japan); ATCC: American Type Culture Collection (Rockville, MD, USA); NCIMB: National Collection of Industrial and Marine Bacteria (Aberdeen, UK); CECT: Colección Española de Cultivos Tipos (Universidad de Valencia, Spain). No specific amplification was detected for any strain. Values in parentheses: number of strains assayed

Bacterial strain	Source	Detection by m-PCR
Vibrio spp. (5) isolated from diseased fish	Laboratory collection	–
Vibrio ordalii (18)	Laboratory collection	–
Vibrio ordalii ATCC 33509	ATCC	–
Vibrio harveyi TW425	Laboratory collection	–
Vibrio splendidus I CPV8.1	Laboratory collection	–
Vibrio splendidus II AZ206	Laboratory collection	–
Vibrio alginolyticus ATCC 17749^T	ATCC	–
Vibrio pelagius I TW487/02	Laboratory collection	–
Vibrio pelagius II RI 152.1	Laboratory collection	–
Aeromonas hydrophila 1404	Laboratory collection	–
Streptococcus parauberis RA9	Laboratory collection	–
Lactococcus garvieae TW94W	Laboratory collection	–
Photobacterium damselae subsp. *piscicida* ATCC 29690	ATCC	–
Photobacterium damselae subsp. *damselae* AZ247.1	Laboratory collection	–
Pseudomonas anguilliseptica CECT 899	CECT	–
Tenacibaculum maritimum NCIMB 2158	NCIMB	–
Tenacibaculum maritimum NCIMB2153	NCIMB	–
Tenacibaculum ovolyticum NBRC 15947	NBRC	–
Tenacibaculum ovolyticum NBRC 15992	NBRC	–
Tenacibaculum mesophilum NBRC 16307^T	NBRC	–
Tenacibaculum mesophilum NBRC 16308	NBRC	–
Tenacibaculum amylolyticum NBRC 16310^T	NBRC	–
Tenacibaculum lutimaris DMS 16505	DMS	–
Rhodococcus qingshengii 79043–3	Laboratory collection	–
Flavobacterium psychrophilum 49418^T	ATCC	–
Flavobacterium columnare ATCC 23462^T	ATCC	–
Yersinia ruckeri CECT 955	CECT	–
Francisella sp. LM-84-F	Laboratory collection	–
Chryseobacterium piscicola CECT 7357^T	CECT	–
Chryseobacterium chaponense Sa 1147-06^T	Laboratory collection	–
Hafnia alvei 15/1403	Laboratory collection	–
Enterobacter cloacae TW 03/03	Laboratory collection	–
Enterobacter aerogenes RPM799.1	Laboratory collection	–
Escherichia coli FV9180	Laboratory collection	–

teine and 1% glucose) was used and incubated at 16°C for 6 d (Mauel et al. 2008). Stock cultures were stored at –80°C in Cryo-bille tubes (AES Laboratory).

DNA extraction

Total DNA was extracted for subsequent analysis using 2 different commercial systems: InstaGene Purification Matrix (Bio-Rad Laboratories) for pure and mixed bacterial cultures and AxyPrep™ Multisource Genomic DNA Miniprep Kit (Axygen Biosciences) for tissue samples. In all cases, DNA purification was performed according to the manufacturer's instructions. The concentration and quality of each DNA sample was examined spectrophotometrically at 260 nm on an Epoch™ Microplate Spectrophotometer. DNA from pure cultures was adjusted with sterile distilled water to a concentration of 50 ± 5 ng μl^{-1}. Of each DNA sample extracted, 1 µl (10 to 5 ng) was used directly for m-PCR amplification, and the remaining DNA sample was stored at –20°C. All experiments were carried out with DNA obtained in 2 independent extractions for each bacterial strain and isolate.

PCR amplification

All PCR amplifications were performed using a Mastercycler personal (Eppendorf) and the commercial kit Ready-To-Go™ PCR beads (GE Healthcare) according to the manufacturer's instructions. This kit included all the reagents needed for the PCR reactions (buffer, nucleotides and *Taq* DNA polymerase), with the exception of the specific primers and DNA template. The sequences of the 4 PCR primer pairs for m-PCR and the expected size of PCR products generated with these primers are shown in Table 3. The PCR annealing temperatures tested ranged from 50 to 60°C. Intensity of the amplicons for each target DNA, as well as the absence of nonspecific bands, was considered in selecting m-PCR conditions. Therefore, 55°C was used as the annealing temperature for all PCRs. The cycling protocol was 1 cycle of 95°C for 3 min, 35 cycles of 95°C for 1 min, 55°C for 1 min and 72°C for 1 min, followed by a final elongation at 72°C for 2 min.

Analysis of PCR products

Aliquots of 10 µl of PCR product were separated on a 1.5% (w/v) agarose gel for 60 min at 100 V in TAE 1× (0.04 M Tris, 0.0001 M EDTA, pH 8.0) electrophoresis buffer, visualized using 0.06 µg ml^{-1} of ethidium bromide (Bio-Rad) and photographed under UV light. A 100 bp DNA ladder (100–1000 bp; Bioron) and GeneRuler™ 100 bp DNA Ladder Plus (100–3000 bp; Fermentas) were used as molecular mass markers. In all cases, negative controls consisting of the same reaction mixture, but with sterile distilled water instead of template DNA, were included in each batch of PCR reactions. The presence of a single product of the appropriate size, identical to the respective reference strains, was considered as a positive result.

Specificity and sensitivity from pure cultures

The specificity of the m-PCR was evaluated using the genomic DNA extracted from 37 related bacteria and an additional 55 different fish pathogens or unrelated bacteria from human (Table 2).

For the evaluation of the analytic sensitivity of m-PCR, the minimum detectable amounts of targeted DNA by m-PCR were detected for *Streptococ-*

Table 3. Specific primers for m-PCR used in the present study

Bacterial species	Target gene	Primer	Sequence (5′–3′)	Amplicon size (bp)	Source
Vibrio anguillarum	*rpoN*	rpoN-ang5′ rpoN-ang3′	GTTCATAGCATCAATGAGGAG GAGCAGACAATATGTTGGATG	519	Gonzalez et al. (2003)
Piscirickettsia salmonis	16S–23S RNA	RTS1 RTS2	TGATTTTATTGTTTAGTGAGAATGA AAATAACCCTAAATTAATCAAGGA	91	Marshall et al. (1998)
Aeromonas salmonicida	*vapA*	AP-1 AP-2	GGCTGATCTCTTCATCCTCACCC CAGAGTGAAATCTACCAGCGGTGC	421	Gustafson et al. (1992)
Streptococcus phocae	16S rRNA	PX1 PXVQ2	GCTAATACCGCATAAGAAGAG CACCCTGTCACTTCTGCTC	900	Hassan et al. (2008) Avendaño-Herrera (2008)

cus phocae ATCC 51973^T, *Aeromonas salmonicida* subsp. *salmonicida* ATCC 33658 or *A. salmonicida* subsp. *achromogenes* ATCC 33659, *Vibrio anguillarum* ATCC 43307 and *Piscirickettsia salmonis* ATCC VR-1361 strains as denoted by Onuk et al. (2010). After DNA extraction, serial dilutions of each DNA were prepared with sterile distilled water at concentrations ranging from 50 ng µl^{-1} to 500 fg µl^{-1}. Aliquots of 10 µl of each dilution of DNA were mixed with the respective aliquots of the other 3 strains. These DNA mixtures were used as template DNA for the m-PCR experiments, and the detection limit for DNA was determined.

For the evaluation of analytic sensitivity of m-PCR from the cultures, based on bacterial cell numbers, separate bacterial suspensions of the *Streptococcus phocae*, *Aeromonas salmonicida*, *Vibrio anguillarum* and *Piscirickettsia salmonis* strains were prepared to contain 10^9 cells ml^{-1} (McFarland Scale 4) and were 10-fold diluted in 0.85% sterile saline solution from 10^8 to 10 cells ml^{-1}. Thus, bacterial mixtures with the different fish pathogens were prepared and used as DNA template for m-PCR sensitivity testing. Chromosomal DNA was extracted as described previously, and CFU ml^{-1} was estimated in all cases by plating onto agar plates. Limits of detection were determined based on presence or absence of PCR products on gels.

Determination of m-PCR sensitivity from spiked fish samples

M-PCR sensitivity was determined employing DNA extracted from *in vitro* spiked spleen, kidney, liver and muscle of juvenile healthy Atlantic salmon as described by Avendaño-Herrera et al. (2004). Each fish sample (mean weight: 16.3 to 12.2 mg) was spiked with 100 µl of one of the bacterial dilutions (from 10^8 to 10^2 cells ml^{-1}) from pure cultures of 4 bacterial species and homogenized in phosphate buffered saline (PBS, pH 7.4) for 60 s. After incubation for 1 h at 18°C, DNA was extracted using AxyPrep™ Multisource Genomic DNA Miniprep Kit according to the manufacturer's instructions. Non-inoculated spiked samples, serving as negative controls, were processed in the same manner but with sterile saline solution instead of bacterial dilutions. For m-PCR, 1 µl of the purified DNA was added as template. Again, limits of detection were determined based on presence or absence of PCR products in gels.

The analytic sensitivity of the m-PCR was also determined using spiked kidney tissue from Atlantic salmon with different relative amounts of *Streptococcus phocae*, *Aeromonas salmonicida* subsp. *salmonicida* or subsp. *achromogenes*, *Vibrio anguillarum* and *Piscirickettsia salmonis.* Here, 100 µl of suspension from each one of 3 bacterial species containing ~10^7 CFU ml^{-1} was mixed with the same amount of a suspension of the fourth pathogen containing 10^4 to 10^5 CFU ml^{-1}. This low concentration was selected considering the sensitivity obtained from the *in vitro* spiked tissues described above.

RESULTS AND DISCUSSION

Rapid diagnosis of outbreak agents is essential for effective control, but current microbiological methods based on culture and biochemical characterization are time-consuming. An m-PCR approach has been successfully applied to detect multiple bacterial pathogens of marine (Kulkarni et al. 2009) and/or freshwater fish (del Cerro et al. 2002, Mata et al. 2004, Altinok et al. 2008, Altinok 2011).

Salmonid rearing in Chile has been seriously threatened by the appearance of a number of diverse bacterial pathogens. In this investigation, an m-PCR method was developed to detect 4 marine pathogens, *Streptococcus phocae, Aeromonas salmonicida*, *Vibrio anguillarum* and *Piscirickettsia salmonis* simultaneously. Firstly, the analytic specificity of the 4 primer sets was determined using the DNA extracted from 37 strains belonging to the 4 targeted species (Table 1), DNA from individual strains being added to reactions. All 4 oligonucleotide primer pairs exclusively amplified the target gene of the specific bacterial pathogen, producing a unique and clear PCR product of the expected length for the corresponding microorganism (Fig. 1). With other strains or isolates used in this study, neither specific nor false-positive bands were detected (Table 2).

The m-PCR condition was optimized for the simultaneous detection of the 4 microorganisms, particularly the annealing temperature, and the best amplification for all primer pairs being obtained at 55°C. The analytic specificity of the m-PCR assay was confirmed using a DNA mixture prepared from the 4 pathogens that gave amplification products of 900 bp for *Streptococcus phocae,* 421 bp for *Aeromonas salmonicida*, 512 bp for *Vibrio anguillarum* and 91 bp for *Piscirickettsia salmonis* (Fig. 1). No amplification was observed when DNA from other taxonomically and/or ecologically related bacteria were combined

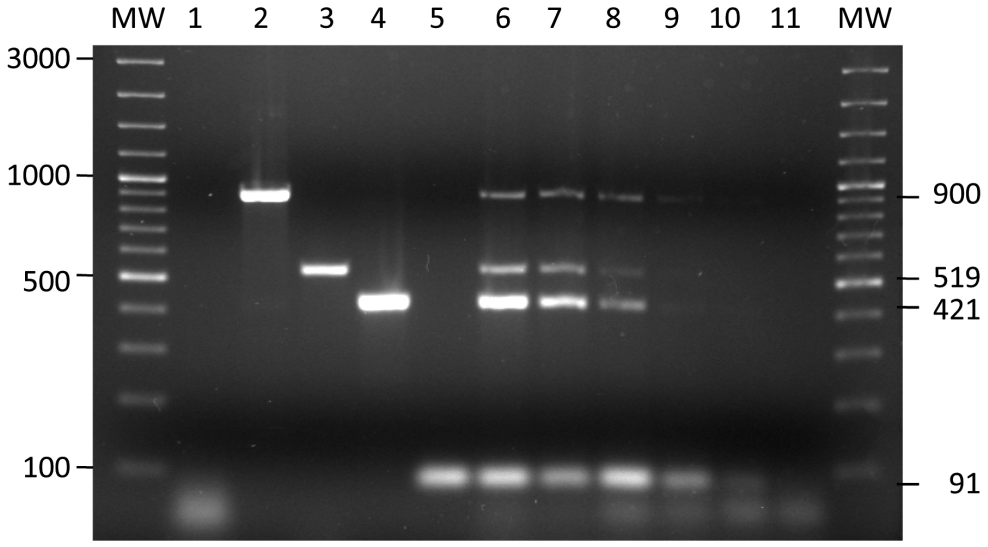

Fig. 1. *Aeromonas salmonicida, Streptococcus phocae, Vibrio anguillarum,* and *Piscirickettsia salmonis.* Amplification products from m-PCR assay developed for the simultaneous detection of streptococcosis, typical and atypical furunculosis, vibriosis and piscirickettsiosis. MW: GeneRuler^{TM} 100bp DNA Ladder Plus (100–3000 bp; Fermentas). Lanes 1 to 5: (1) negative control (no DNA), (2) *S. phocae* ATCC 51973^{T}, (3) *A. salmonicida* subsp. *achromogenes* ATCC 33659, (4) *V. anguillarum* ATCC 43307, (5) *P. salmonis* ATCC VR-1361. Lanes 6 to 11: sensitivity of m-PCR in detecting the 4 fish pathogens from serially diluted (5 ng µl^{-1} to 50 fg µl^{-1}) chromosomal DNA extracted from reference strains of each fish pathogen. Left *y*-axis: position of molecular size marker (bp); right *y*-axis: size of specific amplified products (bp)

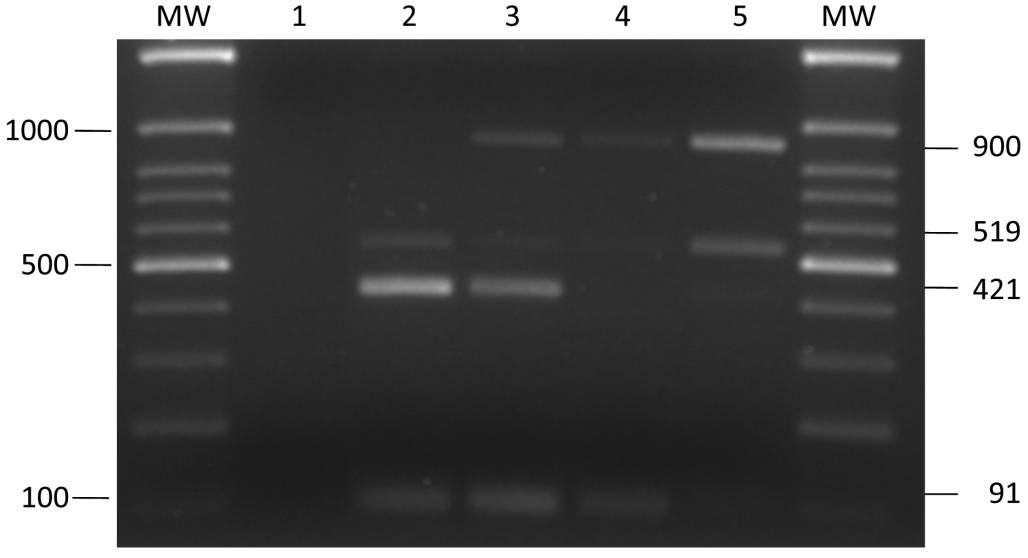

Fig. 2. *Aeromonas salmonicida, Streptococcus phocae*, *Vibrio anguillarum,* and *Piscirickettsia salmonis.* Detection by m-PCR of fish tissue samples containing a high amount of 3 pathogens (10^7 CFU ml^{-1} each bacterium) and low amounts of the fourth pathogen (10^4 to 10^5 CFU ml^{-1}). CFU were estimated by standard plate counts. MW: Axygen 100bp DNA Ladder (100–3000 bp); Lane 1: negative control (no DNA); Lanes 2 to 5: DNA extracted from tissues spiked with (2) *S. phocae* ATCC 51973^{T} at 10^4 CFU ml^{-1}, (3) *V. anguillarum* ATCC 43307 at 10^5 CFU ml^{-1}, (4) *A. salmonicida* subsp. *achromogenes* ATCC 33659 at 10^4 CFU ml^{-1}, and (5) *P. salmonis* ATCC VR-1361 (~150 fg µl^{-1})

together, with the exception of *V. splendidus* and *V. pelagius*, giving a unique, non-specific (i.e. based on size) PCR amplification product of weak intensity (unpubl. data). This can be explained by the high degree of genetic relatedness of the *Vibrio* species, which is defined as a group of strains that share >95% DNA identity in multilocus sequence analysis (MLSA) (Thompson et al. 2009).

The analysis of sensitivity and robustness of the m-PCR protocol for the 4 species was determined by amplification of pure DNA of each reference strain in duplicate. The range of detection using purified DNA was 50 pg µl^{-1} for *Vibrio anguillarum*, 500 fg µl^{-1} for *Piscirickettsia salmonis*, 5 pg µl^{-1} for *Streptococcus phocae* and *Aeromonas salmonicida*, respectively (Fig. 1). In the case of DNA extracted from bacterial suspensions, the average limits in the m-PCR sensitivity for each species were 1.26×10^4 CFU ml^{-1} of *S. phocae*, 3.69×10^5 CFU ml^{-1} of *V. anguillarum* and 5.33×10^4 CFU ml^{-1} of *A. salmonicida*, while the detection limits for the spiked fish tissues was the same, regardless of the tissue type, with $9.03 \pm 1.84 \times 10^5$ CFU g^{-1} for *S. phocae*, $2.64 \pm 0.54 \times 10^7$ CFU g^{-1} for *V. anguillarum*, $3.8 \pm 0.78 \times 10^3$ CFU g^{-1} for *A. salmonicida* and 100 *P. salmonis* cells.

Although the *Piscirickettsia salmonis* strain grew on BFCG agar (Mauel et al. 2008), poorly defined colonies were produced, leading to inaccuracies in estimations of the CFU concentration. In fact, the bacterial count showed that the number of culturable bacteria decreased by 3 log-units (10^5 CFU ml^{-1}) from an initial inoculum of 10^8 cells ml^{-1}. This difficulty leads us to express the sensitivity of *P. salmonis* in DNA concentration units or cells only.

Taking into consideration our detection limit, the simultaneous use of the 4 PCR primer pairs in one PCR resulted in a reduction in analytical sensitivity for *Aeromonas salmonida* and *Vibrio anguillarum* compared to that previously reported in the literature (Gustafson et al. 1992, Gonzalez et al. 2003). In this context, our m-PCR procedure possesses similar detection limits for *Streptococcus phocae* and *Piscirickettsia salmonis* to those previous results obtained by Avendaño-Herrera (2008) and Marshall et al. (1998), respectively. In fact, Avendaño-Herrera (2008) has reported for *S. phocae* detection level on the order of 10^4 CFU ml^{-1} in bacterial suspension and 10^6 CFU g^{-1} in tissues, while for *P. salmonis*, Marshall et al. (1998) suggest that the simple PCR assay is capable of revealing the presence of 10 to 100 *P. salmonis* cells. The m-PCR procedure constitutes a powerful tool for accurate identification of different pathogens from plate cultures as well as from fish tissues (del Cerro et al. 2002, Mata et al. 2004, Altinok et al. 2008, Kulkarni et al. 2009, Onuk et al. 2010). However, an important aspect is that this m-PCR is generally thought to be less sensitive than single PCR due to competition for reaction reagents among the assays making up the multiplex technique, especially if the assays are different in their amplification efficiency or one or more of the target organisms are present in high numbers. In our investigation, the use of high amounts of DNA from 3 bacterial species (10^7 CFU ml^{-1} of each pathogen) had a significant effect on the amplification sensitivity of the other bacterial species only when *A. salmonicida* or *S. phocae* were present in lower concentrations (10^4 to 10^5 CFU ml^{-1}) in the multiplex reaction. In fact, the limits of detection of the *S. phocae* decreased by 1 log-unit (10^6 CFU g^{-1}) when high amounts of amplification of the other 3 target bacterial DNA occurred (Fig. 2). No amplification product was observed when DNA of *A. salmonicida* at lower DNA concentrations (10^5 CFU ml^{-1}) was tested in the presence of higher DNA concentrations (10^7 CFU ml^{-1}) of the other target species. We can speculate that a possible cause can be due to the primer length used and the nucleotide content. Our results are in agreement with Altinok (2011) who noted that the high amounts of DNA from one bacterial species had a significant effect on the amplification sensitivity of the other bacterial species when these are present in lower concentration in the multiplex reaction.

Nevertheless the level of sensitivity is probably sufficient to detect each pathogen in acute infections in fish. For the diagnosis of fish diseases caused by slow-growing bacteria such as *Aeromonas salmonicida* or *Streptococcus phocae*, PCR-based detection is of particular importance because these bacteria can be easily obscured by *Piscirickettsia salmonis* and are therefore likely to be underdiagnosed based on cultivation only. Furthermore, m-PCR assay is more rapid and cost-effective than singleplex PCR. In summary, this m-PCR can be an efficient tool to detect the presence of *S. phocae*, *A. salmonicida*, *Vibrio anguillarum* and *P. salmonis* simultaneously from pure cultures and tissues obtained from clinically diseased fish with high bacterial concentrations. It could therefore be a useful alternative to culture-based methods for the diagnosis of infections in Chilean salmon farms.

Acknowledgements. Funding for this study was provided in part by Grant FONDECYT 1090054 from the Comisión Nacional de Investigación Científica y Tecnológica–CONICYT (Chile) and also by Grant DI-01-10/R from the Universidad Andres Bello.

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Editorial responsibility: Catherine Collins, Aberdeen, UK

Submitted: January 7, 2011; Accepted: July 8, 2011
Proofs received from author(s): November 4, 2011