PCR protocol for detection of *Vibrio ordalii* by amplification of the *vohB* (hemolysin) gene

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ABSTRACT: *Vibrio ordalii* is the causative agent of atypical vibriosis and has the potential to cause severe losses in salmonid aquaculture. To prevent and control outbreaks, a rapid, reproducible, sensitive, and effective diagnostic method is needed. We evaluated a new conventional polymerase chain reaction (PCR) and real-time PCR (qPCR) protocol using a primer set (VohB_Fw–VohB_Rv) designed to amplify a 112 bp fragment flanking the *vohB* gene (coding for hemolysin production), against 24 *V. ordalii* strains isolated from different fish species, the *V. ordalii* type strain, and 42 representative related and unrelated bacterial species. The primer set was species-specific, recognizing all *V. ordalii* strains evaluated, with no cross-reaction with the other bacterial species. A sensitivity of 10^3 copies of the *vohB* gene was obtained with a standard curve. When the VohB_Fw–VohB_Rv qPCR protocol was applied to Atlantic salmon seeded tissues (kidney, liver, spleen, and muscle), the detection limit ranged from 5.27×10^2 to 4.13×10^3 *V. ordalii* CFU ml^{-1}, i.e. 62 to 145 copies of the *vohB* gene, using the previously calculated standard curve. The conventional PCR also detected *V. ordalii*, but the total reaction time was 1 h longer. When the qPCR protocol was applied to naturally infected cage-cultured Atlantic salmon samples, 5 of 8 fish tested positive for *V. ordalii*, but only one of them was diagnosed as positive by direct cultivation on agar. We conclude that the PCR protocol evaluated is fast, specific, and sensitive enough to detect *V. ordalii* in infected tissues and is an important tool for secure diagnosis of atypical vibriosis, and is therefore helpful for the control of the disease through the prompt detection within fish populations.

KEY WORDS: *Vibrio ordalii* · PCR · Atypical vibriosis · Diagnosis · Salmonid fish

INTRODUCTION

Vibriosis is one of the major diseases occurring in marine and brackish fish culture and is characterized by hemorrhagic septicemia. *Vibrio ordalii*, formerly *Listonella anguillarum* biovar II (Schiewe et al. 1981), was isolated for the first time from diseased salmon cultured in coastal waters of the US Pacific Northwest (Harrel et al. 1976). Since its first isolation, *V. ordalii* has been also reported in Japan, Australia, and New Zealand, affecting mainly the culture of several salmonid species (Ransom et al. 1984, Toranzo et al. 1997). From 2004, this pathogen has been reported from southern Chilean cultured popu-

lations of Atlantic salmon *Salmo salar*, Pacific salmon *Oncorhynchus kisutch*, and rainbow trout *O. mykiss* suffering high mortalities (Colquhoun et al. 2004, Silva-Rubio et al. 2008). In Chile, atypical vibriosis is characterized by the presence of skin lesions, hemorrhaging ulcers, and mortality, while the internal signs include pericarditis, peritonitis, multiple necrotic foci in the liver, and signs of systemic septicemia (Bohle et al. 2007).

Detection of the pathogen at an early stage of infection is very important for the prevention and treatment of this atypical vibriosis. The disease is diagnosed presumptively based on the clinical signs of the affected fish, although definitive diagnosis is based on the isolation of bacterial colonies on appropriate media, followed by biochemical tests using conventional tube and plate test procedures. Numerous studies describe *Vibrio ordalii* as a biochemically homogeneous taxon (see reviews by Toranzo et al. 1997, 2005); however, Austin et al. (1997) reported differences by BIOLOG-GN fingerprints and API 20E profiles. Similarly, Colquhoun et al. (2004) demonstrated that Chilean *V. ordalii* isolates share the same biochemical properties present in the previous description of the species (Schiewe et al. 1981), with the exception of the production of acid from trehalose and lack of acid production from mannitol, while Silva-Rubio et al. (2008) reported that all studied strains failed to hydrolyze gelatin. Therefore, the disadvantage of these microbiological methods, besides being time-consuming, is that the biochemical testing can be ambiguous; therefore, molecular detection methods are becoming more preferred.

At present, the use of polymerase chain reaction (PCR)-based techniques such as real-time or quantitative PCR (qPCR) has become an important diagnostic tool in the detection of different pathogens affecting aquatic animals (Purcell et al. 2011). Furthermore, the qPCR method, using technologies such as a 5′ nuclease assay (e.g. Taqman assay) or the dye intercalation assay (e.g. SYBR-green), improves the accuracy and sensitivity of traditional PCR and allows the target gene to be detected and quantified directly in different matrices, which is often faster than conventional PCR. Ribosomal DNA genes (e.g. 16S ribosomal RNA) are a common choice for the development of qPCR assays (Karatas et al. 2008, Orieux et al. 2011, Fringuelli et al. 2012) due to their widespread use in phylogenetic analysis of bacteria. However, gene targets specific to *Vibrio ordalii* such as the 16S rRNA, internal transcribed spacer (ITS), or 5S rRNA sequences are not sufficiently discriminatory to distinguish *V. ordalii* from other closely related *Vibrio* species, especially *V. anguillarum* (MacDonell & Colwell 1984, Pillidge & Colwell 1988, Ito et al. 1995, Wiik et al. 1995, Fernández & Avendaño-Herrera 2009). Therefore, there is great interest in finding a unique nucleotide sequence in the *V. ordalii* genome (Naka et al. 2011) useful for the rapid detection of this pathogen.

Among the currently available *Vibrio ordalii* gene sequences in the EMBL database, we selected the *vohB* gene coding for hemolysin (a putative virulence factor) production as a PCR target because it discriminates *V. ordalii* from other sequences of phylogenetically related species. Here we present the first report on the use of a PCR method for the specific diagnosis and quantification of atypical vibriosis caused by *V. ordalii*.

MATERIALS AND METHODS

Bacterial strains and growth conditions

In total, 24 *Vibrio ordalii* strains isolated from different Atlantic salmon farms during 2003 to 2010 were used in this study (Table 1). The reference strains of *V. ordalii* ATCC 33509[T] and *V. anguillarum* ATCC 43305 (serotype O1), ATCC 43306 (serotype O2), and ATCC 43307 (serotype O3) from the American Type Culture Collection were included for comparative purposes. The identity of each isolate was confirmed as *V. ordalii* by using the following criteria: Gram-negative motile bacilli, cytochrome oxidase positive, fermenting glucose, sensitive to the vibriostatic agent 0/129, and no growth onto thiosulfate-citrate-bile salts-sucrose agar (TCBS). Additional biochemical reactions were performed as described by Schiewe et al. (1981) and also using a miniaturized API 20E system (bioMèrieux) (Silva-Rubio et al. 2008). Biochemical profiles of our isolates were compared with those of the *V. ordalii* type strain.

In addition, reference strains of other pathogens from fish and mammals also obtained from outbreaks were included as negative controls to develop and optimize the conventional PCR and qPCR assay (Table 1). The identity of each isolate was confirmed by standard phenotypical and microscopic techniques as reported by MacFaddin (1980) and also in some cases using PCR-based analysis or serological assays (Tapia-Cammas et al. 2011).

All strains were routinely cultivated on tryptone soya agar or broth supplemented with 1% (w/v) sodium chloride (TSA-1 or TSB-1, respectively) at

Table 1. *Vibrio ordalii* strains and other bacterial species included in this study. 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; DMSZ: German Collection of Microorganisms and Cell Cultures

Bacterial strain	Source	PCR/qPCR detection
Vibrio ordalii ATCC 33509[T]	ATCC	+/+
Vibrio ordalii (24)[a]	Laboratory collection	+/+
Vibrio anguillarum 43305 - serotype O1	ATCC	–/–
Vibrio anguillarum 43306 - serotype O2	ATCC	–/–
Vibrio anguillarum 43307 - serotype O3	ATCC	–/–
Isolates from diseased fish *Vibrio* sp. (5)[b]	Laboratory collection	–/–
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	DMSZ	–/–
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	–/–[c]
Escherichia coli FV9180	Laboratory collection	–/–[c]
Aeromonas salmonicida subsp. *achromogenes* ATCC 33659	ATCC	–/–
Streptococcus phocae ATCC 51973[T]	ATCC	–/–
Piscirickettsia salmonis ATCC VR-1361	ATCC	–/–

[a]The number of strains assayed is indicated in parentheses
[b]These *Vibrio* spp. were not confirmed as *V. ordalii* by biochemical and phenotypic traits. The molecular techniques allowed the classification of *Vibrio* species into the *Splendidus* clade
[c]These microorganims gave a unique, non-specific (i.e. based on size) PCR amplification product

20°C for 24 to 72 h, except for some fish pathogens included in Table 1 that were grown on the following media: Columbia sheep blood medium (AES Laboratory) for the Gram-positive bacteria; *Flexibacter maritimus* Medium (FMM; Pazos et al. 1996) for the *Tenacibaculum* species; Austral-TSHem medium (Yáñez et al. 2013) for *Piscirickettsia salmonis*; TYES medium (Valdebenito & Avendaño-Herrera 2009) for *Flavobacterium* and *Chryseobacterium* species and Luria-Bertani medium for *Escherichia coli* and *Enterobacter* species. All were cultured according to the temperature and period of incubation optimal for each pathogen. Stock cultures were maintained frozen at −80°C in Criobilles tubes (AES Laboratory) or TSB-1 and 15% (v/v) glycerol.

DNA extraction

Total DNA was extracted from pure bacterial cultures employing the InstaGene Purification Matrix (Bio-Rad Laboratories). 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 using an Epoch™ Microplate Spectrophotometer. DNA from pure cultures was adjusted with sterile distilled water using a ScanDrop analyzer (AnalytikJena), and 1 µl of each DNA suspension (4.74 ± 1.48 ng µl^{-1}) was used directly for each qPCR when pure culture was tested. The remaining DNA samples were stored at −20°C. All experiments were carried out with DNA obtained from 2 independent extractions for each bacterial strain and isolate.

Design of the primer set

The sequence of the *Vibrio ordalii* ATCC 33509^T *vohB* gene (accession no AB210836) was retrieved from GenBank and compared with the closest relatives employing the Basic Local Alignment Search Tool (BLAST) software (www.ncbi.nlm.nih.gov/blast/ Blast.cgi). On the basis of the alignment, the most variable regions in comparison to different *Vibrio* species were chosen. Using Primer 3 software (Rozen & Skaletsky 2000), a primer pair that would work effectively as qPCR primers was designed for the *vohB* gene. Primers were synthesized by Invitrogen™ and designated as VohB_Fw (5′-CCT TGC CAC TAT TCG AGT CAT-3′) and VohB_Rv (5′-CGT ACA GAA TAT GGG CAT CG-3′), which give an amplification product of 112 bp. In addition, primer specificity was assessed for possible similarity to sequences present in the GenBank database using BLAST.

Conventional PCR and optimization of qPCR conditions

Primers were first tested against the *Vibrio ordalii* isolates using a Mastercycler personal apparatus (Eppendorf) and the commercial kit Ready-To-Go™ PCR beads (GE Healthcare) according to the manufacturer's instructions. This kit included all of the reagents needed for the PCR reactions (buffer, nucleotides, and *Taq* DNA polymerase), with the exception of the 10 µM of each specific primer and the template DNA. The PCR annealing temperatures tested ranged from 50 to 60°C. Intensity of the amplicon for the target DNA, as well as the absence of nonspecific bands, was considered in selecting PCR conditions.

The cycling protocol was 1 cycle of 95°C for 10 min, 40 cycles of 95°C for 15 s, 55°C for 15 s, and 72°C for 15 s, followed by a final elongation at 72°C for 5 min. Amplified PCR products were analyzed on 2% (w/v) agarose gels with TAE (0.04 M Tris, 0.0001M EDTA, pH 8.0) electrophoresis buffer, and the expected size of the amplicons was confirmed by comparing the known DNA molecular mass markers (10–200 Ultra Low Range DNA Ladder, Fermentas), which were visualized with a UV transilluminator after staining with ethidium bromide (0.06 µg ml^{-1}). After determining the specificity of the primer pairs (Table 1), the detection limit using purified DNA from the *Vibrio ordalii* ATCC 33509^T strain and the isolate Vo-LM-06 was also determined.

In addition, qPCR assays were performed in 25 µl reactions using the SensiMixTM SYBR Kit (Bioline), which included all reagents needed for the PCR reactions, except 1 µl of the DNA and each primer (2 µM) as described above. Samples were amplified using a Stratagene Mx3000P QPCR System, and the PCR conditions were the same as for conventional PCR, apart from the final elongation step. Finally, a melting curve was constructed from 55 to 95°C. The qPCR amplicons were confirmed by electrophoresis in 2% Seakem LE agarose gel and ethidium bromide staining.

Nucleic acid sequencing

Nucleotide sequences amplified using PCR and qPCR were confirmed using the ABI PRISM Kit reaction with AmpliTaq DNA polymerase on an ABI PRISM 310 sequencer (Applied Biosystems) according to the manufacturer's recommendations. The sequence of the 4 Chilean *Vibrio ordalii* isolates and the type strain ATCC 33509^T were aligned and compared with the *V. ordalii* (AB210836) sequence obtained from BLAST (GenBank).

Construction of the standard curve for DNA quantification

To create *Vibrio ordalii* qPCR standards, the *vohB* gene was amplified as reported previously. The amplification product obtained with the primer pairs VohB_Fw and VohB_Rv was cloned into pGEM®-T

Easy Vector Systems (Promega), and once its specificity was confirmed by sequencing, the plasmid constructed was named the vohB-112 genome. Plasmid DNA was isolated using an Axygene Plasmid Miniprep kit according to the manufacturer's protocol. The purified vohB-112 genome was quantified with a ScanDrop analyzer (AnalytikJena) and serially diluted in DNAase/RNAase-free water to give a range of 1 to 10^{10} copies of genome equivalent ml^{-1}. One µl of each dilution was used for qPCR, in triplicate and in 2 independent qPCR assays, to create a standard curve to quantify *V. ordalii* DNA, while stock plasmid was stored at −80°C. The detection limit was determined as the lowest concentration (within the linear range) that produced an amplification signal in all 3 replicates. The DNA plasmid concentration was converted to genomic equivalents based on the assumption that single copies of the *vohB* target sequence are present in the genome. A linear regression line analysis was performed, and the coefficient of determination (R^2) was calculated. In addition, conventional PCR was also performed as described above on the plasmid dilutions, and the qPCR and conventional amplicons were confirmed by electrophoresis in 2% Seakem LE agarose gel and ethidium bromide staining.

Specificity and sensitivity from pure and mixed cultures

The specificity of the primer set designed was evaluated using total DNA extracted from pure colonies of 24 Chilean *Vibrio ordalii* isolates and the fish pathogens included in Table 1. The sensitivity of the primers VohB_Rv and VohB_Fw, using pure cultures, was evaluated as described by Avendaño-Herrera et al. (2004). Briefly, pure colonies of 2 strains representative of the *V. ordalii* species (Chilean isolate Vo-LM-06 and the type strain ATCC 33509[T]) were selected from TSA-1 plates, adjusted to contain 10^9 cells ml^{-1} (McFarland Scale 4), and serially diluted in 0.85% sterile saline solution from 10 to 10^8 cells ml^{-1} (i.e. from 4 to 4.13 × 10^8 CFU ml^{-1} for the type strain and from 5 to 5.27 × 10^8 CFU ml^{-1} for Vo-LM-06).

To determine the usefulness of the primer sets to amplify template *Vibrio ordalii* DNA from mixed cultures, bacterial suspensions of *Piscirickettsia salmonis* ATCC VR-1361, *Aeromonas salmonicida* subsp. *achromogenes* ATCC 33659, and *Streptococcus phocae* ATCC 51973[T] were employed. Thus, bacterial mixtures of *V. ordalii* with 3 other different fish pathogens were prepared at equal proportions (1:1:1:1) for qPCR sensitivity testing. To each dilution of the *V. ordalii* strain (equivalent to 4.13 × 10^6 CFU ml^{-1} and 5.27 × 10^6 CFU ml^{-1} for the type strain and isolate Vo-LM-06, respectively), 100 µl of each bacterial suspension that contained approximately 10^7 cells ml^{-1} was added.

CFUs of the other fish pathogens were estimated by plating each dilution onto appropriate media and counting the bacterial colonies produced. The average for *Aeromonas salmonicida* subsp. *achromogenes* ATCC 33659 and *Streptococcus phocae* ATCC 51973[T] was 1.36 × 10^6 and 6.34 × 10^6 CFU ml^{-1}, respectively. Although the *Piscirickettsia salmonis* strain grew on Austral-TSHem agar (Yáñez et al. 2013), estimation of the CFU concentration was difficult and the number of culturable bacteria decreased by 3 log-units (10^4 CFU ml^{-1}) from an initial inoculum of 10^7 cells ml^{-1}.

Conditions for DNA extraction and qPCR amplification were the same as described above. The detection level was determined based on the presence or absence of a fluorescence signal as well as the PCR products on gels.

Determination of qPCR sensitivity from spiked fish samples

To ensure that the *Vibrio ordalii*-specific primer set could efficiently detect/quantify *V. ordalii* directly from Atlantic salmon samples, qPCR sensitivity was also determined employing DNA extracted with an AxyPrep™ Multisource Genomic DNA Miniprep Kit (Axygen Biosciences) from *in vitro* seeded kidney (17.1 ± 3.1 mg), liver (28.6 ± 3.8 mg), spleen (16.8 ± 2.6 mg), and muscle (29.8 ± 6.6 mg) of juvenile healthy Atlantic salmon as previously described by Avendaño-Herrera et al. (2004).

In brief, each fish sample (from 16.8 to 29.8 mg) was seeded with 100 µl of different bacterial dilutions from pure cultures of isolate Vo-LM-06 (from 5.27 ± 0.56 × 10^2 to 5.27 ± 0.56 × 10^9 CFU ml^{-1}) and the type strain ATCC 33509[T] (from 4.13 ± 0.23 × 10^2 to 4.13 ± 0.23 × 10^9 CFU ml^{-1}), homogenized for 60 s in microtubes with a micropestle containing phosphate-buffered saline (pH 7.4) at a final volume of 1 ml; DNA was extracted from the full volume. Non-inoculated tissue samples, with sterile saline solution added instead of bacterial dilutions, were used as negative controls and were processed in the same manner. All results were assessed by repetition in 2 independent qPCR reactions, in which DNA

obtained from 2 different extractions was employed. For qPCR, 1 µl of the purified DNA was added as template. Limits of detection were determined based on the presence or absence, in triplicate reactions from 2 independent DNA extracts, of fluorescence signal, as well as the PCR products on gels.

Detection of *Vibrio ordalii* in Atlantic salmon from naturally infected tissues

Detection of *Vibrio ordalii* using the primer set VohB_Fw–VohB_Rv was evaluated in 8 fish samples, which were obtained from a farm in which a vibriosis outbreak occurred. Tissue samples were collected as pools of organs (kidney, spleen and liver) from each fish ensuring an amount of 20 mg of total pooled tissue and processed for DNA extraction as described above. In addition, 5 pools of tissue were obtained from Atlantic salmon cultured at a local farm that was registered free from vibriosis. All pooled tissues were analyzed by qPCR assay to confirm the presence or absence of *V. ordalii*. Positive controls were obtained from fish experimentally inoculated with the *V. ordalii* type strain. The reference gene used for amplification control was β-actin (Evenhuis & Cleveland 2012). The bacterial culture method was used with all fish sampled from the vibriosis outbreak and negative control sites to look for the presence of *V. ordalii* from each internal organ (kidney, spleen, liver) by direct streaking onto TSA-1 plates.

Statistical analysis

Serially diluted DNA was quantified to generate a calibration curve. For each standard, vohB-112 copy numbers were plotted against the cycle number at which the fluorescence signal increased above the background or threshold value (C_t value). Amplification of the specific sequence of the *vohB* gene for *Vibrio ordalii* was confirmed by the melting temperature of the amplicon and by gel electrophoresis. Finally, the Pearson product-moment correlation coefficient (r) was used to examine the relation between C_t values and log concentrations of purified plasmids.

RESULTS AND DISCUSSION

Salmonid rearing in Chile has been seriously threatened by the appearance of a number of diverse bacterial pathogens such as *Vibrio ordalii*

(Ibieta et al. 2011). Rapid diagnosis of outbreak agents is essential for effective control, but current microbiological methods based on culture and biochemical identification of *V. ordalii* are time-consuming and tedious. In addition, *V. ordalii* often appears in mixed infections with *Piscirickettsia salmonis*, increasing the possibility of misdiagnosis. Recently, DNA-based methods have been successfully applied to detecting multiple bacterial pathogens of marine (Campbell & Wright 2003, Karatas et al. 2008, Crisafi et al. 2011, Fringuelli et al. 2012) and/or freshwater salmonids (Sandell & Jacobson 2011, Orieux et al. 2011, Bastardo et al. 2012). Here we developed a conventional PCR and qPCR protocol to detect *V. ordalii*.

Design of the primer set

In the last 2 decades, bacterial studies have been enriched with molecular chronometers, for example rRNA genes (5S, 16S, and 23S), to reconstruct bacterial phylogenies but also to be used as taxonomic markers for identification (Thompson et al. 2004). However, *Vibrio ordalii* and *V. anguillarum* show a high degree of similarity of their overall DNA sequence, as revealed by chromosomal DNA–DNA pairing (Schiewe et al. 1981). In addition, DNA comparison of 5S rRNA gene sequences revealed a close relationship between *V. anguillarum* and *V. ordalii*, differing only in 7 of 120 bp (MacDonell & Colwell 1984, Pillidge & Colwell 1988, Ito et al. 1995). Similarly, *V. ordalii* is indistinguishable from *V. anguillarum* on the basis of the 16S rRNA gene sequence (Wiik et al. 1995). Indeed, our BLAST analysis of the *V. ordalii* 16S gene sequence confirmed 99% identity with the closely related *V. anguillarum*. All of these gene sequences are insufficiently divergent to warrant the design of *V. ordalii* species-specific PCR primers using that gene as a target. Therefore, several other genes, e.g. *vohB*, *recA*, *chitinase A*, and *hsp60*, were analyzed to identify a suitable target gene for PCR assays to detect *V. ordalii*. The *vohB* sequence from *V. ordalii* 33509[T] (accession number AB210836) shared 92% identity with *V. anguillarum* hemolysin toxin *vah3* gene (accession numbers CP002284 and AB189396, respectively), and displayed sufficient variation to design a putatively specific PCR/qPCR assay. Interestingly, the same difficulty in the detection of other *Vibrio* species such as *V. anguillarum*, *V. alginolyticus*, *V. parahaemolyticus*, and *V. metschnikovii* was solved using different genes such as the ones that encode the σ54 factor,

DNA gyrase subunit B protein (*gyrB*), thermostable direct hemolysin (*tdh*), and initiation factor 3 (*infC*) (Gonzalez et al. 2003, Zhou et al. 2007, Tyagi et al. 2009, Cao et al. 2010).

The chosen gene codes for an unknown *Vibrio ordalii* hemolysin, which showed 92% identity with hemolysin toxin *vah3* gene (accession number AB189396) present in the *V. anguillarum* chromosome (Rodkhum et al. 2006). Bacterial hemolysins are cytolytic toxins generally considered to be virulence factors due to their ability to affect erythrocytes and other cell types (Rowe & Welch 1994). Nevertheless, the relationship between hemolysin production and pathogenicity of this bacterium is still controversial. Kodama et al. (1984) reported that *V. ordalii* strain N7802 did not produce hemolysin, but recently the complete genome sequence of *V. anguillarum* 775 and the draft genomic sequences of 2 other *V. anguillarum* strains (96F and RV22,) as well as *V. ordalii* ATCC 33509[T] have been determined and analyzed (Naka et al. 2011), establishing that all hemolysin genes are conserved in these *Vibrio* isolates, except for *vah4* which is absent in *V. ordalii* ATCC 33509[T] and *V. anguillarum* RV22.

Specificity of PCR reaction primers

The specificity of the primers VohB_Fw and VohB_Rv was assayed by using 25 *Vibrio ordalii* strains, including the *V. ordalii* type strain and 42 other bacterial species. After conventional PCR amplification, all *V. ordalii* strains studied produced a unique and clear PCR product of the expected 112 bp length. Subsequent sequence analyses of the amplicons from the 4 Chilean *V. ordalii* isolates revealed 100% identity at the nucleotide level within the 112 nucleotides of the *vohB* coding region, and all sequences were in complete agreement with the *vohB* sequence for the type strain ATCC 33509[T] previously deposited in GenBank (accession number AB210836). This implies that the chosen *V. ordalii* hemolysin gene is conserved, although further studies are needed to confirm whether it corresponds to the hemolysin toxin *vah3* gene or to another gene. No amplification from any other non-target bacterial DNA belonging to related species such as *V. anguillarum* was observed with the primers used, nor from the other fish pathogens tested (Table 1), with the exception of *Enterobacter aerogenes* RPM799.1 and *Escherichia coli* FV9180 which gave a unique, non-specific (i.e. approximately 140 and 190 bp in size) PCR amplification product of weak intensity (data

not shown). A similar non-specific result has been reported for *Photobacterium damselae* by Osorio et al. (1999), who noted a non-specific PCR amplification product produced by a *V. splendidus* PCR assay. According to Cao et al. (2010), if the PCR bands on the gel are weak, and the sizes of the products are similar, then an accurate judgment is difficult to make by naked eye. However, here the non-specific products were easy to distinguish on gels based on their size. Fluorescence real-time technology provides advantages of both easier discrimination of different products and sensitivity. When total DNA from pure cultures was used as qPCR template, each cultured *V. ordalii* strain tested here was detected at C_t values 19.1 ± 0.5 and at a melting temperature of 79.5 ± 0.3°C. No fluorescent signal was detected in the nuclease-free water blank used as a negative control or with any non-*V. ordalii* strains (no C_t), with the exception of *E. aerogenes* RPM799.1 and *E. coli* FV9180, which gave a C_t of 27.49 and 29.73 with melting temperatures of 83.44 and 78.33°C, respectively. Cao et al. (2010) noted that different characteristic dissociation curves with different melting temperatures can be used to accurately determine species-specific amplification. We have not investigated the observed non-specific amplification, but we speculate that a possible origin may be the existence of a putative bacterial hemolysin in these microorganisms. Sequencing of the products is needed to confirm this hypothesis.

Finally, it is pertinent to note that the 2 species of the family *Enterobacteriaceae* have different ecological habitats and distinct host specificities compared to *Vibrio ordalii*, while the other fish pathogen species assayed were not detected by the *V. ordalii*-specific assay. In fact, despite 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 (Thompson et al. 2004), the VohB_Fw and VohB_Rv primer pair did correctly and specifically amplify *V. ordalii.*

However, the total conventional PCR procedure from the point of DNA extraction to observation in an agarose gel took at least 1 h longer in comparison with the qPCR approach. This does not include time required to prepare the gel, which can be done during the PCR reaction and therefore does not add to 'time to result.' It is important to note that although the conventional PCR allows only the detection of the pathogen and not the true quantification of *Vibrio ordalii*, this method is the most commonly used in the Chilean diagnostic laboratories as most of them do not have a qPCR thermocycler.

Standard curve establishment and range of detection

Conventional PCR cannot be used for quantitative purposes, whereas qPCR has proven useful for the reliable detection and quantification of very low numbers of pathogen gene copies in host organisms (Purcell et al. 2011). Therefore, a reproducible standard curve, over a wide range of *vohB* copy numbers, was developed for *Vibrio ordalii* to determine its concentration in samples. Repeated runs of the 10-fold dilutions of the plasmid were positive for all samples with a minimum amount of target material of 10^3 bacterial DNA copies per reaction (Fig. 1). Although theoretically, qPCR can detect a single copy of any given gene per reaction (Guy et al. 2003, Harms et al. 2003, Saikaly et al. 2007), in practice sensitivity becomes limiting below 100 gene copies per mixture (Qi et al. 2001, He & Jiang 2005, Seurinck et al. 2005, Yáñez et al. 2005). The slope of the calibration curve was 3.2484, indicating an amplification efficiency of 99% with an R^2 of 0.9984 (Fig. 1). Thus, measurements within and between runs showed a high level of reproducibility, which was demonstrated by the repetitions of the amplifications in triplicate and at least 2 independent qPCR assays.

Although qPCR is commonly more sensitive than conventional PCR (Adams & Thompson 2011), in the present study, when DNA extracted from plasmid was used as template in conventional PCR amplification, the limits of detection of the *vohB* gene were identical to the level of amplification obtained with qPCR assays (Fig. 2). It has been reported that sensitivity of PCR varies depending on the size of amplicons, and generally primers yielding smaller amplicons will be able to detect lower levels of target organisms (Otta et al. 1999, Hossain et al. 2001). In our study, the size of the amplification product was
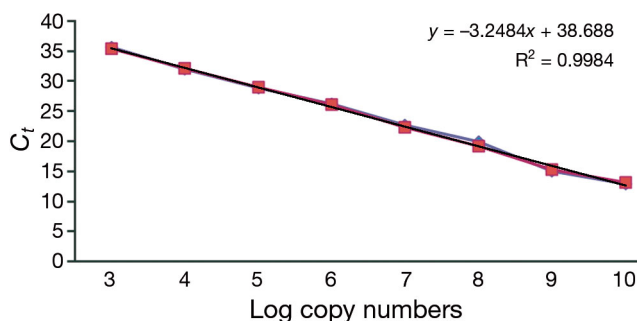
small (112 bp) and the PCR thermal program for the qPCR and conventional PCR were the same, and may have been the reason for the identical results.

qPCR sensitivity from pure and mixed cultures and infected fish samples

The qPCR sensitivity from DNA extracted from different samples is shown in Table 2. In the case of DNA extracted from pure bacterial suspensions, the detection limits in the qPCR reactions for isolate Vo-
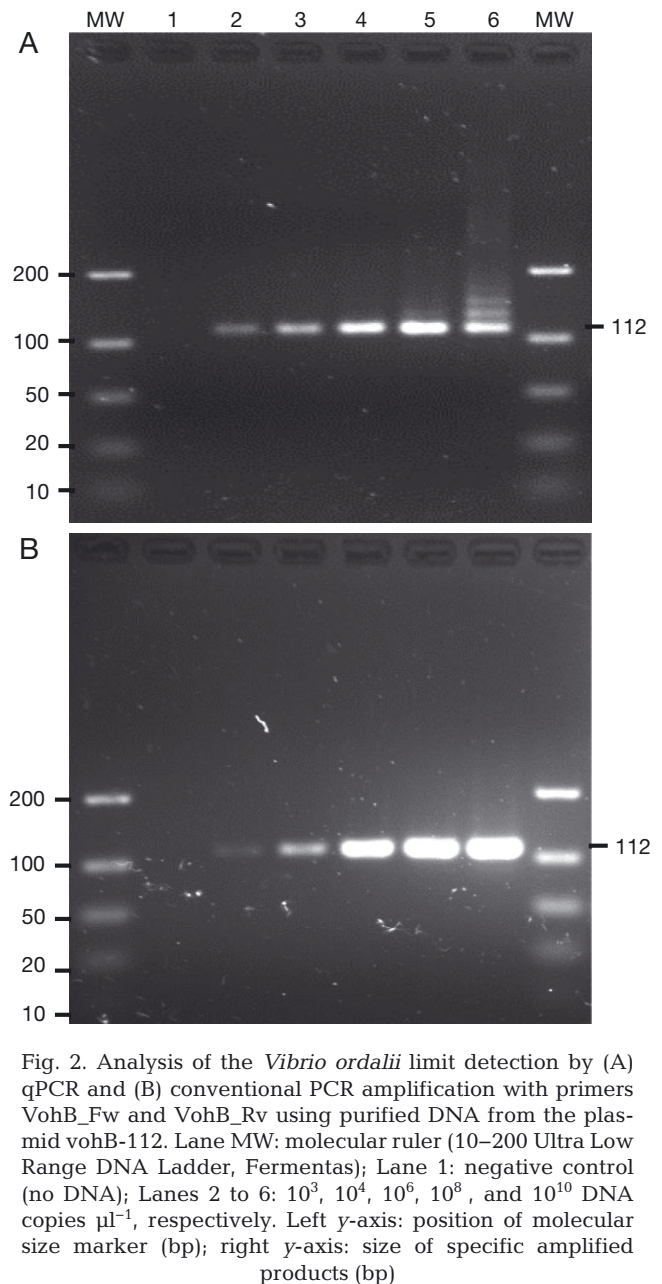

Fig. 2. Analysis of the *Vibrio ordalii* limit detection by (A) qPCR and (B) conventional PCR amplification with primers VohB_Fw and VohB_Rv using purified DNA from the plasmid vohB-112. Lane MW: molecular ruler (10–200 Ultra Low Range DNA Ladder, Fermentas); Lane 1: negative control (no DNA); Lanes 2 to 6: 10^3, 10^4, 10^6, 10^8, and 10^{10} DNA copies μl^{-1}, respectively. Left *y*-axis: position of molecular size marker (bp); right *y*-axis: size of specific amplified products (bp)

Fig. 1. Standard curve of qPCR to detect *Vibrio ordalii*. Relationship between C_t values and amount of plasmid vohB-112 genome equivalents is on a log scale. Blue and red lines: replicates; black line: regression

Table 2. Sensitivity obtained with the qPCR protocol using pure and mixed cultures, as well as fish samples seeded with 2 strains representative of the *Vibrio ordalii* species. C_t: threshold cycle number. CFU values are given ± SE

	ATCC 33509[T]			Vo-LM-06		
	CFU (ml^{-1} or g^{-1})	C_t	Log copy number	CFU (ml^{-1} or g^{-1})	C_t	Log copy number
Culture						
Pure	4130 ± 230	31.66	2.16	527 ± 56	32.86	1.79
Mixed	4130 ± 230	31.42	2.24	527 ± 56	33.96	1.81
Fish samples						
Kidney	$2.42 ± 0.07 × 10^6$	29.48	2.83	$3.08 ± 0.02 × 10^6$	32.94	2.31
Liver	$1.44 ± 0.61 × 10^5$	31.08	2.34	$1.84 ± 1.48 × 10^4$	33.26	1.98
Spleen	$2.46 ± 0.88 × 10^6$	29.66	2.78	$3.14 ± 2.17 × 10^5$	31.94	2.08
Muscle	$1.39 ± 0.08 × 10^6$	29.13	2.94	$1.77 ± 0.85 × 10^5$	31.24	2.29

LM-06 and the type strain ATCC 33509[T] were 5.27 × 10^2 and 4.13 × 10^3 CFU ml^{-1}, respectively. When the number of equivalent gene copies was calculated for the C_t value obtained, using the standard curve, the levels of detection for the *vohB* gene ranged from 62 to 145 copies (Table 2). Although the sensitivity and specificity of the PCR detection for target bacteria can be reduced in mixed cultures (Lee et al. 1995), when we compared the results obtained with pure cultures and the mixed culture containing 10^7 cells of *Piscirickettsia salmonis* ATCC VR-1361, *Aeromonas salmonicida* subsp. *achromogenes* ATCC 33659, and *Streptococcus phocae* ATCC 51973[T], the detection limits of the qPCR were not affected (Table 2). These non-*Vibrio* species were selected because they are some of the pathogens reported in Chile (Avendaño-Herrera 2011) which could interfere with the detection of *V. ordalii* from infected fish.

In the case of the qPCR from spiked fish samples, DNA extracted from fish samples used as negative controls did not yield any amplification product, while the minimum level of detection was 1.84 ± 1.48 × 10^4 and 1.44 ± 0.61 × 10^5 *Vibrio ordalii* CFU g^{-1} when spiked with a pure bacterial suspension of Vo-LM-06 or ATCC 33509[T], respectively. The C_t values obtained from the analysis of tissue samples were converted using the previously generated standard curve, giving a minimum level of detection of 95 and 218 copies of the *vohB* gene, respectively. The lowest detection limits were observed when the liver was tested. It is important to note that the limit of detection of the same samples

using conventional PCR were identical to the level of detection obtained with qPCR assay.

In this work, we tested whether *Vibrio ordalii* could be detected by qPCR assay directly from naturally infected cage-cultured Atlantic salmon from a pool of different organs. The qPCR assay gave positive results with the correct melting curve temperature for *V. ordalii* in 5 of the 8 fish samples, while for the other 3 samples as well as for the negative controls, no amplification for *V. ordalii* was obtained. The bacterial loads determined in the different samples of naturally infected fish ranked from 435 to 1300 DNA copies per reaction (C_t values of 28.57 to 30.1). It is important to note that the analysis of the β-actin indicated the presence of genomic DNA in all samples at a level indicative of successful extraction (C_t = 18.1 ±1.3), with exception of the reaction tube without DNA. In addition, all results were confirmed using agarose gel electrophoresis, showing all positive
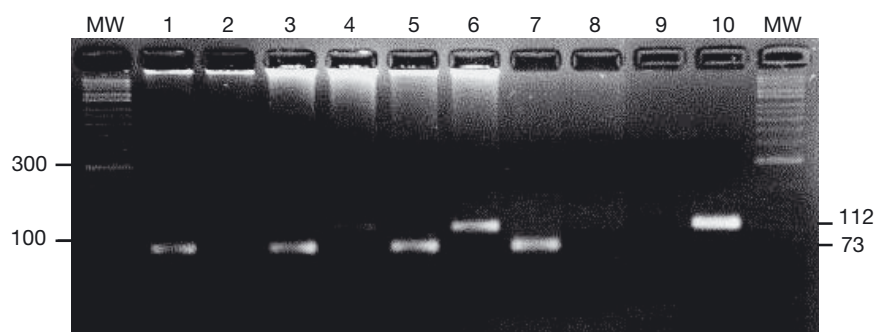

Fig. 3. Detection of *Vibrio ordalii* from fish samples obtained from naturally infected cage-cultured Atlantic salmon using the qPCR protocols with VohB_Fw and VohB_Rv (Lanes 2, 4, 6, 8 and 10) and β-actin forward and β-actin reverse (Lanes 1, 3, 5, 7 and 9) primer sets. Lanes: MW, HyperLadder II (50–2000 bp ladder, Bioline); 1 to 6, DNA extracted from fish samples; 7 and 10, positive control; 8 and 9, negative control (no DNA). Numbers on the left indicate the position of molecular size marker (kb). Numbers on the right indicate the size of the specific amplified products (bp)

samples giving a unique and clear amplification product of the expected 112 bp length (Fig. 3).

Attempts to culture *Vibrio ordalii* from the naturally infected fish directly streaked onto TSA-1 plates was successful in 1 of the 8 cases analyzed (with a C_t = 30.1), and the remaining fish were diagnosed as negative for the presence of *V. ordalii* by classical plate-culturing methods. Obtaining pure cultures of *V. ordalii* from external or internal tissues is difficult, due to the characteristic slow growth of this pathogen which allows other opportunistic species to overgrow it. In addition, growth at a suboptimal temperature (20°C) as was employed in our study might affect the growth of *V. ordalii* in plates as denoted by Poblete-Morales et al. (2013). Unfortunately, our PCR studies were already underway before Poblete-Morales et al. (2013) published those results. Our findings indicate that the qPCR results obtained were true positives despite that the plate cultures were negative, due to mortalities with clinical signs of vibriosis subsequently being observed in the cages.

In general, a broad range of bacterial cell numbers (from 1 up to 10^8 CFU) can be measured using qPCR (Nadkarni et al. 2002). Taking into consideration our results, the limit of detection was less sensitive than those achieved in other studies for fish pathogens (Suzuki & Sakai 2007, Jung et al. 2010, Keeling et al. 2012). Our study also showed that when the PCR protocol was used with DNA extracted from seeded tissue samples, the sensitivity level was lower than that observed with pure and mixed cultures (Table 2), probably due to presence of host DNA and undefined inhibitors of PCR (Wilson 1997). However, the levels of sensitivity are probably sufficient to detect acute *Vibrio ordalii* infections in fish.

The quantification limits obtained by testing DNA extracted from culture or host tissues seeded with *Vibrio ordalii* Vo-LM-06 showed a slight increase in the level of detection calculated for the *vohB* gene when compared with the detection limit established for the vohB-112 plasmid standard curve (10^3 bacterial DNA copies). However, although the qPCR generated consistent positive results from DNA samples, there was no consistency with the equivalence in the copy numbers detecting as few as 10^2 plasmid copies in replicate reactions. We speculate that the qPCR assay could be on the edge of the detection limit (10^2 to 10^3). Additional standard curves made in fish tissues seeded with vohB-112 plasmid are needed to confirm this hypothesis. Therefore, the diagnostic to *V. ordalii* cannot be used quantitatively for samples with fewer than 10^2 targets. Based on these qPCR results, we recommend considering a *V. ordalii* DNA

sample as positive if $C_t \leq 33$ and if the amplification of the specific target gene is confirmed by a melting temperature of the amplicon equal to 79.5 ± 0.3°C or the observation of the correct sized product (112 bp) on an electrophoresis gel.

Clinical laboratories are increasing the use of PCR procedures to complement or replace classic diagnostic assays, often in the context of prevention programs or to ensure the identification of a pathogen (Cunningham 2002). The main advantage of the *Vibrio ordalii* qPCR is that it can be a useful tool to quantify *V. ordalii* in the samples and is faster to perform, but the findings obtained for conventional PCR constitute an additional value to this study, since it is relevant for many diagnostic laboratories that do not have a real-time machine (Bastardo et al. 2012).

In conclusion, the new primer pair described here has provided a rapid and specific procedure for the detection and quantification of *Vibrio ordalii* by conventional PCR and qPCR. This is the first time *V. ordalii* has been detected by SYBR Green I real-time PCR, which provides the simplest and most economical format for detecting and quantifying PCR products (De Medici et al. 2003). Moreover, these protocols proved to be useful not only in pure or mixed bacterial suspensions but also in artificially seeded fish tissues with values of $1.84 \pm 1.48 \times 10^4$ *V. ordalii* CFU g^{-1} of tissue or the equivalent to up to 95 copies of the *vohB* gene being detected. Therefore, these molecular tools represent a useful alternative to the microbiological approach for the rapid and specific diagnosis of *V. ordalii* infection in fish farms.

LITERATURE CITED

Adams A, Thompson KD (2011) Development of diagnostics for aquaculture: challenges and opportunities. Aquacult Res 42:93–102

Austin B, Austin DA, Blanch AR, Cerda M and others (1997) A comparison of methods for the typing of fish-pathogenic *Vibrio* spp. Syst Appl Microbiol 20:89–101

Avendaño-Herrera R (2011) Enfermedades infecciosas del cultivo de salmónidos en Chile y el mundo. NIVA, Puerto Varas

Avendaño-Herrera R, Magariños B, Toranzo AE, Beaz R, Romalde JL (2004) Species-specific polymerase chain

reaction primer sets for the diagnosis of *Tenacibaculum maritimum* infection. Dis Aquat Org 62:75–83

Bastardo A, Ravelo C, Romalde JL (2012) Highly sensitive detection and quantification of the pathogen *Yersinia ruckeri* in fish tissues by using real-time PCR. Appl Microbiol Biotechnol 96:511–520

Bohle H, Kjetil F, Bustos P, Riofrío A, Peters C (2007) Fenotipo atípico de *Vibrio ordalii*, bacteria altamente patogénica aislada desde salmón del Atlántico cultivado en las costas marinas del sur de Chile. Arch Med Vet 39: 43–52

Campbell MS, Wright AC (2003) Real-time PCR analysis of *Vibrio vulnificus* from oysters. Appl Environ Microbiol 69:7137–7144

Cao J, Xu J, Zheng Q, Yan P (2010) Rapid detection of *Vibrio metschnikovii* in aquatic products by real-time PCR. Folia Microbiol (Praha) 55:607–613

Colquhoun DJ, Aase IL, Wallace C, Baklien Å, Gravningen K (2004) First description of *Vibrio ordalii* from Chile. Bull Eur Assoc Fish Pathol 24:185–188

Crisafi F, Denaro R, Genovese M, Cappello S, Mancuso M, Genovese L (2011) Comparison of 16SrDNA and toxR genes as target for detection of *Vibrio anguillarum* in *Dicentrarchus labrax* kidney and liver. Res Microbiol 162:223–230

Cunningham CO (2002) Molecular diagnosis of fish and shellfish diseases: present status and potential use in disease control. Aquaculture 206:19–55

De Medici D, Croci L, Delibato E, Di Pasquale S, Filetici E, Toti L (2003) Evaluation of DNA extraction methods for use in combination with SYBR Green I real-time PCR to detect *Salmonella enterica* serotype *enteritidis* in poultry. Appl Environ Microbiol 69:3456–3461

Evenhuis JP, Cleveland BM (2012) Modulation of rainbow trout (*Oncorhynchus mykiss*) intestinal immune gene expression following bacterial challenge. Vet Immunol Immunopathol 146:8–17

Fernández J, Avendaño-Herrera R (2009) Analysis of 16S–23S intergenic spacer regions of *Vibrio anguillarum* and *Vibrio ordalii* strains isolated from fish. FEMS Microbiol Lett 299:184–192

Fringuelli E, Savage PD, Gordon A, Baxter EJ, Rodger HD, Graham DA (2012) Development of a quantitative real-time PCR for the detection of *Tenacibaculum maritimum* and its application to field samples. J Fish Dis 35:579–590

Gonzalez SF, Osorio CR, Santos Y (2003) Development of a PCR-based method for the detection of *Listonella anguillarum* in fish tissues and blood samples. Dis Aquat Org 55:109–115

Guy RA, Payment P, Krull UJ, Horgen PA (2003) Real-time PCR for quantification of *Giardia* and *Cryptosporidium* in environmental water samples and sewage. Appl Environ Microbiol 69:5178–5185

Harms G, Layton AC, Dionisi HM, Gregory IR and others (2003) Real-time PCR quantification of nitrifying bacteria in a municipal wastewater treatment plant. Environ Sci Technol 37:343–351

Harrel LW, Novotny AJ, Schiewe MJ, Hodgins HO (1976) Isolation and description of two vibrios pathogenic to Pacific salmon in Puget Sound, Washington. Fish Bull 74: 447–449

He JW, Jiang S (2005) Quantification of enterococci and human adenoviruses in environmental samples by real-time PCR. Appl Environ Microbiol 71:2250–2255

Hossain MSA, Otta SK, Karunasagar I, Karunasagar I (2001)

Detection of white spot syndrome virus (WSSV) in wild captured shrimp and in non-cultured crustaceans from shrimp ponds in Bangladesh by polymerase chain reaction. Fish Pathol 36:93–95

Ibieta P, Tapia V, Venegas C, Hausdorf M, Takle H (2011) Chilean salmon farming on the horizon of sustainability: review of the development of a highly intensive production, the ISA crisis and implemented actions to reconstruct a more sustainable aquaculture industry. In: Sladonja B (ed) Aquaculture and the environment — a shared destiny. InTech, Rijeka, doi: 10.5772/30678

Ito H, Uchida I, Sekizaki T, Terakado N (1995) A specific oligonucleotide probe based on 5S rRNA sequences for identification of *Vibrio anguillarum* and *Vibrio ordalii.* Vet Microbiol 43:167–171

Jung MY, Chang YH, Kim W (2010) A real-time PCR assay for detection and quantification of *Lactococcus garvieae.* J Appl Microbiol 108:1694–1701

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

Keeling SE, Johnston C, Wallis R, Brosnahan CL, Gudkovs N, McDonald WL (2012) Development and validation of real-time PCR for the detection of *Yersinia ruckeri.* J Fish Dis 35:119–125

Kodama H, Moustafa M, Ishiguro S, Mikami T, Izawa H (1984) Extracellular virulence factors of fish *Vibrio*: relationships between toxic material, hemolysin, and proteolytic enzyme. Am J Vet Res 45:2203–2207

Lee CY, Pan SF, Chen CH (1995) Sequence of a cloned pR72H fragment and its use for detection of *Vibrio parahaemolyticus* in shellfish with the PCR. Appl Environ Microbiol 61:1311–1317

MacDonell MT, Colwell RR (1984) Nucleotide base sequence of *Vibrionaceae* 5S rRNA. FEBS Lett 175:183–188

MacFaddin JF (1980) Biochemical tests for identification of medical bacteria, 2nd edn. Waverly Press, Baltimore, MD

Nadkarni MA, Martin FE, Jacques NA, Hunter N (2002) Determination of bacterial load by real-time PCR using a broad-range (universal) probe and primers set. Microbiology 148:257–266

Naka H, Dias GM, Thompson CC, Dubay C, Thompson FL, Crosa JH (2011) Complete genome sequence of the marine fish pathogen *Vibrio anguillarum* harboring the pJM1 virulence plasmid and genomic comparison with other virulent strains of *V. anguillarum* and *V. ordalii.* Infect Immun 79:2889–2900

Orieux N, Bourdineaud JP, Douet DG, Daniel P, Le Henaff M (2011) Quantification of *Flavobacterium psychrophilum* in rainbow trout, *Oncorhynchus mykiss* (Walbaum), tissues by qPCR. J Fish Dis 34:811–821

Osorio CR, Collins MD, Toranzo AE, Barja JL, Romalde JL (1999) 16S rRNA gene sequence analysis of *Photobacterium damselae* and nested PCR method for rapid detection of the causative agent of fish pasteurellosis. Appl Environ Microbiol 65:2942–2946

Otta SK, Shubha G, Joseph B, Chakraborty A, Karunasagar I, Karunasagar I (1999) Polymerase chain reaction (PCR) detection of white spot syndrome virus (WSSV) in cultured and wild crustaceans in India. Dis Aquat Org 38: 67–70

Pazos F, Santos Y, Macías AR, Núñez S, Toranzo AE (1996) Evaluation of media for the successful culture of *Flexibacter maritimus.* J Fish Dis 19:193–197

Pillidge CJ, Colwell RR (1988) Nucleotide sequence of the 5S rRNA from *Listonella* (*Vibrio*) *ordalii* ATCC 33509 and *Listonella* (*Vibrio*) *tubiashii* ATCC 19105. Nucleic Acids Res 16:3111

Poblete-Morales M, Irgang R, Henríquez-Núñez H, Toranzo AE, Kronvall G, Avendaño-Herrera R (2013) *Vibrio ordalii* antimicrobial susceptibility testing — modified culture conditions required and laboratory-specific epidemiological cut-off values. Vet Microbiol 165:434–442

Purcell MK, Getchell RG, McClure CA, Gerver KA (2011) Quantitative polymerase chain reaction (PCR) for detection of aquatic animal pathogens in a diagnostic laboratory setting. J Aquat Anim Health 23:148–161

Qi Y, Patra X, Liang L, Williams E, Rose S, Redkar RJ, Delvecchio VG (2001) Utilization of the rpoB gene as a specific chromosomal marker for real-time PCR detection of *Bacillus anthracis.* Appl Environ Microbiol 67:3720–3727

Ransom DP, Lannan CN, Rohovec JS, Fryer JL (1984) Comparison of histopathology caused by *Vibrio anguillarum* and *Vibrio ordalii* and three species of Pacific salmon. J Fish Dis 7:107–115

Rodkhum C, Hirono I, Crosa JH, Aoki T (2006) Multiplex PCR for simultaneous detection of five virulence hemolysin genes in *Vibrio anguillarum.* J Microbiol Methods 65:612–618

Rowe GE, Welch RA (1994) Assays of hemolytic toxins. Methods Enzymol 235:657–667

Rozen S, Skaletsky H (2000) Primer3 on the WWW for general users and for biologist programmers. Methods Mol Biol 132:365–386

Saikaly PE, Barlaz MA, De los Reyes FL III (2007) Development of quantitative real-time PCR assays for detection and quantification of surrogate biological warfare agents in building debris and leachate. Appl Environ Microbiol 73:6557–6565

Sandell TA, Jacobson KC (2011) Comparison and evaluation of *Renibacterium salmoninarum* quantitative PCR diagnostic assays using field samples of Chinook and coho salmon. Dis Aquat Org 93:129–139

Schiewe MH, Trust TJ, Crosa JH (1981) *Vibrio ordalii* sp. nov.: a causative agent of vibriosis in fish. Curr Microbiol 6:343–348

Seurinck S, Defoirdt T, Verstraete W, Siciliano SD (2005) Detection and quantification of the human-specific HF183 *Bacteroides* 16S rRNA marker with real-time PCR for assessment of human faecal pollution in freshwater. Environ Microbiol 7:249–259

Silva-Rubio A, Acevedo C, Magariños B, Jaureguiberry B,

Toranzo AE, Avendaño-Herrera R (2008) Antigenic and molecular characterization of *Vibrio ordalii* strains isolated from Atlantic salmon (*Salmo salar*) in Chile. Dis Aquat Org 79:27–35

Suzuki K, Sakai DK (2007) Real-time PCR for quantification of viable *Renibacterium salmoninarum* in chum salmon *Oncorhynchus keta*. Dis Aquat Org 74:209–223

Tapia-Cammas D, Yáñez A, Arancibia G, Toranzo AE, Avendaño-Herrera R (2011) Multiplex PCR for the detection of *Piscirickettsia salmonis, Vibrio anguillarum, Aeromonas salmonicida* and *Streptococcus phocae* in Chilean marine farms. Dis Aquat Org 97:135–142

Thompson FL, Iida T, Swings J (2004) Biodiversity of vibrios. Microbiol Mol Biol Rev 68:403–431

Toranzo AE, Santos Y, Barja JL (1997) Immunization with bacterial antigens: *Vibrio* infections. In: Gudding R, Lillehaug A, Midtlyng PJ, Brown F (eds) Developments in biologicals, Vol 90: Fish vaccinology. Karger, Basel, p 93–105

Toranzo AE, Magariños B, Romalde JL (2005) A review of the main bacterial fish diseases in mariculture systems. Aquaculture 246:37–61

Tyagi A, Saravanan V, Karunasagar I, Karunasagar I (2009) Detection of *Vibrio parahaemolyticus* in tropical shellfish by SYBR green real-time PCR and evaluation of three enrichment media. Int J Food Microbiol 129:124–130

Valdebenito S, Avendaño-Herrera R (2009) Phenotypic, serological and genetic characterization of *Flavobacterium psychrophilum* strains isolated from salmonids in Chile. J Fish Dis 32:321–333

Wiik R, Stackebrandt E, Valle O, Daae FL, Rødseth OM, Andersen K (1995) Classification of fish-pathogenic vibrios based on comparative 16S rRNA analysis. Int J Syst Bacteriol 45:421–428

Wilson IG (1997) Inhibition and facilitation of nucleic acid amplification. Appl Environ Microbiol 63:3741–3751

Yáñez MA, Carrasco-Serrano C, Barbera VM, Catalan V (2005) Quantitative detection of *Legionella pneumophila* in water samples by immunomagnetic purification and real-time PCR amplification of the dotA gene. Appl Environ Microbiol 71:3433–3441

Yáñez AJ, Silva H, Valenzuela K, Pontigo JP and others (2013) Two novel blood-free solid media for the culture of the salmonid pathogen *Piscirickettsia salmonis.* J Fish Dis 36:587–591

Zhou S, Hou Z, Qin Q (2007) Development of a SYBR Green I real-time PCR for quantitative detection of *Vibrio alginolyticus* in seawater and seafood. J Appl Microbiol 103:1897–1906