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-

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 103 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 × 102 to 4.13 × 103 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

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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 is 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 & Avena-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<sup>T</sup> 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 O/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 (bioMérieux) (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 soy agar or broth supplemented with 1% (w/v) sodium chloride (TSA-1 or TSB-1, respectively) at
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.

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

<table>
<thead>
<tr>
<th>Bacterial strain</th>
<th>Source</th>
<th>PCR/qPCR detection</th>
</tr>
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<tbody>
<tr>
<td><em>Vibrio ordalii</em> ATCC 33509&lt;sup&gt;T&lt;/sup&gt;</td>
<td>ATCC</td>
<td>+/+</td>
</tr>
<tr>
<td><em>Vibrio ordalii</em> (24)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Laboratory collection</td>
<td>+/+</td>
</tr>
<tr>
<td><em>Vibrio anguillarum</em> 43305 - serotype O1</td>
<td>ATCC</td>
<td>–/–</td>
</tr>
<tr>
<td><em>Vibrio anguillarum</em> 43306 - serotype O2</td>
<td>ATCC</td>
<td>–/–</td>
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<tr>
<td><em>Vibrio anguillarum</em> 43307 - serotype O3</td>
<td>ATCC</td>
<td>–/–</td>
</tr>
<tr>
<td>Isolates from diseased fish <em>Vibrio</em> sp. (5)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Laboratory collection</td>
<td>–/–</td>
</tr>
<tr>
<td><em>Vibrio harveyi</em> TW425</td>
<td>Laboratory collection</td>
<td>–/–</td>
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<tr>
<td><em>Vibrio splendidus</em> I CPV8.1</td>
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<td>–/–</td>
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<tr>
<td><em>Vibrio splendidus</em> II AZ206</td>
<td>Laboratory collection</td>
<td>–/–</td>
</tr>
<tr>
<td><em>Vibrio algolynoticus</em> ATCC 17749&lt;sup&gt;T&lt;/sup&gt;</td>
<td>ATCC</td>
<td>–/–</td>
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<tr>
<td><em>Vibrio pelagius</em> I TW487/02</td>
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<td>–/–</td>
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<tr>
<td><em>Vibrio pelagius</em> II RI 152.1</td>
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<td>–/–</td>
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<tr>
<td><em>Aeromonas hydrophila</em> 1404</td>
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<td>–/–</td>
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<tr>
<td><em>Streptococcus parauberis</em> RA9</td>
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<td>–/–</td>
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<tr>
<td><em>Lactococcus garvieae</em> TW94W</td>
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<tr>
<td><em>Photobacterium damselae</em> subsp. <em>piscicida</em> ATCC 29690</td>
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</tr>
<tr>
<td><em>Photobacterium damselae</em> subsp. <em>damselae</em> AZ247.1</td>
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<td>–/–</td>
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<tr>
<td><em>Pseudomonas anguilliseptica</em> CECT 899</td>
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<td>–/–</td>
</tr>
<tr>
<td><em>Tenacibaculum maritimum</em> NCIMB 2158</td>
<td>NCIMB</td>
<td>–/–</td>
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<tr>
<td><em>Tenacibaculum maritimum</em> NCIMB2153</td>
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<tr>
<td><em>Tenacibaculum ovolyticum</em> NBRC 15947</td>
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<tr>
<td><em>Tenacibaculum ovolyticum</em> NBRC 15992</td>
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<td><em>Tenacibaculum mesophilum</em> NBRC 16307&lt;sup&gt;T&lt;/sup&gt;</td>
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<td><em>Tenacibaculum mesophilum</em> NBRC 16308</td>
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<tr>
<td><em>Tenacibaculum amylyticum</em> NBRC 16310&lt;sup&gt;T&lt;/sup&gt;</td>
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<td><em>Tenacibaculum lutimaris</em> DMS 16505</td>
<td>DMSZ</td>
<td>–/–</td>
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<td><em>Rhodococcus qingshengii</em> 79043-3</td>
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<td>–/–</td>
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<tr>
<td><em>Flavobacterium psychrophilum</em> 49418&lt;sup&gt;T&lt;/sup&gt;</td>
<td>ATCC</td>
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<tr>
<td><em>Flavobacterium columnare</em> ATCC 23462&lt;sup&gt;T&lt;/sup&gt;</td>
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<tr>
<td><em>Yersinia ruckeri</em> CECT 955</td>
<td>CECT</td>
<td>–/–</td>
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<td><em>Francisella sp.</em> LM-84-F</td>
<td>Laboratory collection</td>
<td>–/–</td>
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<tr>
<td><em>Chryseobacterium piscicola</em> CECT 7357&lt;sup&gt;T&lt;/sup&gt;</td>
<td>CECT</td>
<td>–/–</td>
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<tr>
<td><em>Chryseobacterium chaponense</em> Sa 1147-06&lt;sup&gt;T&lt;/sup&gt;</td>
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<td>–/–</td>
</tr>
<tr>
<td><em>Hafnia alvei</em> 15/1403</td>
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<td>–/–</td>
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<tr>
<td><em>Enterobacter cloacae</em> TW 03/03</td>
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<td>–/–</td>
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<tr>
<td><em>Enterobacter aerogenes</em> RPM799.1</td>
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<td>–/–</td>
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<tr>
<td><em>Escherichia coli</em> FV9180</td>
<td>Laboratory collection</td>
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<tr>
<td><em>Aeromonas salmonicida</em> subsp. <em>achromogenes</em> ATCC 33659</td>
<td>ATCC</td>
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<tr>
<td><em>Streptococcus phocae</em> ATCC 51973&lt;sup&gt;T&lt;/sup&gt;</td>
<td>ATCC</td>
<td>–/–</td>
</tr>
<tr>
<td><em>Piscirickettsia salmonis</em> ATCC VR-1361</td>
<td>ATCC</td>
<td>–/–</td>
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</tbody>
</table>

<sup>a</sup>The number of strains assayed is indicated in parentheses

<sup>b</sup>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

<sup>c</sup>These microorganisms gave a unique, non-specific (i.e. based on size) PCR amplification product
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 33509T 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 Tag 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 33509T strain and the isolate Vol-LM-06 was also determined.

In addition, qPCR assays were performed in 25 µl reactions using the SensiMix™ 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 33509T 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/RNase-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.

**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 V. 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 V. ordalii DNA from mixed cultures, bacterial suspensions of Piscirickettsia salmonis ATCC VR-1361, Aeromonas salmonicida subsp. achronogenes 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. achronogenes 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 V. 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 × 10^6 CFU ml^{-1}) and the type strain ATCC 33509^T (from 4.13 ± 0.23 × 10^2 to 4.13 ± 0.23 × 10^6 CFU ml^{-1}), homogenized for 60 s in microtubes with a micropastele 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 & Cleve-
land 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 (Ct 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 Ct 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* CP002284 (accession numbers 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. mettchnikovii* 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 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 33509T 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 33509T 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 33509T 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 \( \text{voHB} \) 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 \( \text{voHB} \) 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-
LM-06 and the type strain ATCC 33509T 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 Ct 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 51973T, 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 33509T, respectively. The Ct 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 (Ct 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 (Ct = 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

<table>
<thead>
<tr>
<th>Culture</th>
<th>CFU (ml⁻¹ or g⁻¹)</th>
<th>C_t</th>
<th>Log copy number</th>
<th>CFU (ml⁻¹ or g⁻¹)</th>
<th>C_t</th>
<th>Log copy number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pure</td>
<td>4130 ± 230</td>
<td>31.66</td>
<td>2.16</td>
<td>527 ± 56</td>
<td>32.86</td>
<td>1.79</td>
</tr>
<tr>
<td>Mixed</td>
<td>4130 ± 230</td>
<td>31.42</td>
<td>2.24</td>
<td>527 ± 56</td>
<td>33.96</td>
<td>1.81</td>
</tr>
<tr>
<td>Kidney</td>
<td>2.42 ± 0.07 × 10^6</td>
<td>29.48</td>
<td>2.83</td>
<td>3.08 ± 0.02 × 10^6</td>
<td>32.94</td>
<td>2.31</td>
</tr>
<tr>
<td>Liver</td>
<td>1.44 ± 0.61 × 10^5</td>
<td>31.08</td>
<td>2.34</td>
<td>1.84 ± 1.48 × 10^4</td>
<td>33.26</td>
<td>1.98</td>
</tr>
<tr>
<td>Spleen</td>
<td>2.46 ± 0.88 × 10^6</td>
<td>29.66</td>
<td>2.78</td>
<td>3.14 ± 2.17 × 10^5</td>
<td>31.94</td>
<td>2.08</td>
</tr>
<tr>
<td>Muscle</td>
<td>1.39 ± 0.08 × 10^6</td>
<td>29.13</td>
<td>2.94</td>
<td>1.77 ± 0.85 × 10^5</td>
<td>31.24</td>
<td>2.29</td>
</tr>
</tbody>
</table>

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
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 ($10^5$ 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.

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