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Fast detection of *Vibrio* species potentially pathogenic for mollusc

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ABSTRACT

Vibrio tasmaniensis, *Vibrio splendidus* and *Vibrio neptunius* species were distributed worldwide and associated with aquaculture and have been reported as the cause of diseases in aquatic organisms. Polyphasic analyses for bacterial identification are not feasible for routine diagnostic because of the time involved. The aim of this study is to design three PCR primer sets that can assist with fast detection of these species. They were designed from the 16S ribosomal RNA gene, and PCR conditions were found. Each PCR test successfully identified all the tested strains of each target species. The combined specificity of *V. tasmaniensis* and *V. splendidus* primer sets offered the best coverage (86%) in terms of separating target organisms from other related species. The primer set of *V. tasmaniensis* showed a lower sensitivity limit (500 fg of DNA) than the *V. splendidus* set (1 pg) and both sets gave positive amplification using homogenized tissues from inoculated clams, with 10^2 and 10^4 cfu/g of clam, respectively. The primer set of *V. neptunius* was highly specific, showing only cross-reaction with *V. parahaemolyticus* species from 44 tested species. Its sensitivity limit was 100 pg of DNA. A small number of biochemical tests were proposed concurrently with the PCR to differentiate the cross-reacting bacteria. The time of detection of the three tested species was reduced and the further affected animals can be diagnosed in a rapid fraction of time. The detection of virulent strains of *V. tasmaniensis* pointed to the risk of mollusc culture outbreaks.

1. Introduction

Vibrio tasmaniensis, *Vibrio splendidus* and *Vibrio neptunius* were the predominant species found by us associated with the culture of oysters and clams (Guisande et al., 2008). These species have been distributed worldwide and were associated with diseases in aquatic organisms in different countries. So, over the last few years, *V. splendidus* has been mainly associated with disease in fish (both larvae and juvenile specimens) (Angulo et al., 1994a, b; Santos et al., 1997; Gatesoupe et al., 1999; Thomson et al., 2005; Sitjà-Bobadilla et al., 2007; Reid et al., 2009), molluscs (Sugumar et al., 1998; Le Roux et al., 2002; Gay et al., 2004; Gómez-León et al., 2005; Garnier et al., 2007; Guisande et al., 2008) and gorgonians (Hall-Spencer et al., 2007). *V. neptunius* and *V. tasmaniensis*, the species commonly considered to be non-pathogenic (Thompson et al., 2003a, 2003b) have been associated with disease in fish and molluscs and sea cucumbers and gorgonian, respectively (Austin et al., 2005; Prado et al., 2005; Hall-Spencer et al., 2007; Guisande et al., 2008; Deng et al., 2009). The main reason for increase in infections lies in intensive cultures, since it contributes to concentrating the pathogenic and opportunistic species.

V. tasmaniensis and *V. splendidus* are two closely related species included in the *V. splendidus*-related group. This group shares a high level of homogeneity (Macián et al., 2001; Montes et al., 2003, 2006; Le Roux et al., 2004; Thompson et al., 2005) and several molecular methods have been previously proposed to differentiate between

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them. Guisande et al. (2008) used a combination of ribotyping and sequencing of 16S rRNA gene, while Thompson et al. (2005) suggested the analysis of the three loci *recA*, *rpoA*, *pyrH* and Le Roux et al. (2004) the *gyrB* gene. The combination of genotypic and phenotypic analyses was also previously reported for the identification of the *V. neptunius* species (Thompson et al., 2003a; Guisande et al., 2008). Neither of these techniques is feasible for routine diagnostic laboratories.

The design of molecular methods for fast detection of pathogenic *Vibrio* species would be essential for improving industrial culture production. Specific PCR primers that amplify gene coding for bacterial 16S rRNA have been widely used as a target showing useful results (Saulnier et al., 2000; Oakey et al., 2003; Avendaño-Herrera et al., 2004).

The aim of this study is to evaluate the specificity and sensitivity of three PCR primer sets designed from the 16S rDNA sequence to assist with a fast detection of *V. tasmaniensis*, *V. splendidus* and *V. neptunius* species by using colonies obtained from a laboratory collection. The usefulness of the PCR tests was evaluated on homogenized tissues from experimentally inoculated clams. In addition, the risk of outbreaks and virulence of *V. tasmaniensis* on *Venerupis rhomboides* was estimated.

2. Material and methods

2.1. Bacterial strains

The *Vibrio* strains used in this study are shown in Table 2. Several strains were previously associated with Galician aquaculture, were isolated from mollusc and turbot and were identified by phenotypic ribotyping and 16S rRNA sequence analysis (Farto et al., 2003, 2006; Montes et al., 2006; Guisande et al., 2008). Other types and reference strains were obtained from various culture collections and maintained in the laboratory.

The original *Vibrio* strains were grown in tryptic soy broth (Cultimed, Barcelona, Spain) supplemented with 2% (w/v) NaCl (Panreac) (TSB-2) and 15% (v/v) of glycerol (Panreac, Madrid, Spain) at 22 °C for 48 h and stored at −80 °C. Freeze-dried pure cultures of *Vibrio* strains (Table 2) were routinely cultivated on tryptic soy agar (Cultimed) supplemented with 2% (w/v) NaCl (Panreac, Barcelona, Spain) (TSA-2) at 22 °C for 48 h. *Aeromonas*, *Pseudomonas* and *Tenacibaculum* strains were cultivated and conserved as was reported for the Spanish collection of type culture (CECT).

2.2. Selection of target priming sequences

A pair of oligonucleotide probes for each species, *V. tasmaniensis* (VTS and VT), *V. splendidus* (VTS and VS) and *V. neptunius* (VN1 and VN2) were selected by us and synthesized by Invitrogen (UK). The sequence, position and size are shown in Table 1.

The sequences of variable regions of the 16S rRNA genes of *Vibrio* strains in this study were determined, aligned and compared with all available bacterial 16S rRNA sequences in order to search for specific target sites. Previously reported 16S sequences from *V. tasmaniensis*, *V. splendidus*, *V. neptunius* and other Vibrionaceae species were extracted from GenBank.

Table 1
Primers used in PCR.

Primer	Sequence
Common primer of *V. tasmaniensis* and *V. splendidus*	
76-95f (VTS)	5′ GAGCGGAAACGACACTAACA 3′ (20 bp)[a]
Primer of *V. tasmaniensis*	
475-496r (VT)	5′ GCA**GCTATTAAC**TACACACCCT 3′ (22 bp)
Primer of *V. splendidus*	
482-503r (VS)	5′ AAGAGATAGC**GCTATTAAC**GCT 3′ (22 bp)
Primer of *V. neptunius*	
99-113f (VN-1)	5′ AAAGCCTTCGGGTGG 3′ (15 bp)
443-460r (VN-2)	5′ ACACCACCTTCCTCACTG 3′ (18 bp)

[a] Primer size; in black shared region by both primers; f, forward; r, reverse.

2.3. PCR optimization and primers specificity

The DNA was amplified from colonies of each strain with the pair of primers of each species: VTS/VT; VTS/VS; VN1/VN2, firstly using 30 cycles and an annealing temperature of 55 °C. The 100 μL PCR-reaction mixture contained sterile distilled water, dNTP mix (25 mM each; Invitrogen), 200 ng of each primer set (Invitrogen) and 5 u/μL of Taq DNA polymerase (Bioline, UK). Amplification was performed in a Techgene Thermal cycle (Techne, UK). The protocol was 94 °C for 4 min, followed by 30 cycles of 94 °C for 30 sec, 55 °C for 1 min, 72 °C for 2 min, with a final extension of 72 °C for 7 min. PCR amplified fragments were purified on Microspin columns (Amersham Pharmacia Biotech, UK) and visualized in 1% agarose gel and staining with ethidium bromide; a molecular mass marker (digoxigenin-labelled λ phage digested with *Hin*dIII; Invitrogen) was also added.

A temperature gradient assay (Eppendorf mastercycler gradient) was conducted for optimization and specificity, for each primer set. The annealing temperature interval assayed for *V. tasmaniensis* and *V. splendidus* was 65–75 °C, and 48–62 °C for *V. neptunius*. Amplification was conducted by using the standard conditions described above, except that 40 cycles for *V. neptunius* were used.

The most favoured conditions and the maximum specific temperature were considered to be those which, in combination, showed high reproducibility over a number of replicate tests and showed an amplified product from each species tested and as few (if any) of the other species tested. These PCR assays were repeated three times with several strains to ensure reproducibility.

The specificity of these probes was evaluated by using 12, 8 and 7 strains of *V. tasmaniensis*, *V. splendidus* and *V. neptunius*, respectively and between 56 and 85 strains belonging to 42 other species (Table 2). In addition, colonies re-isolated from inoculated clams (*Ven. rhomboides*) for strains of *V. tasmanienisis* (see experimental infections section) were tested for specificity. The re-