Quantification of Vibrio penaeicida, the etiological agent of Syndrome 93 in New Caledonian shrimp, by real-time PCR using SYBR Green I chemistry

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Abstract:

Shrimp farming is a small but growing industry in New Caledonia. Since 1993, “Syndrome 93” has been affecting New Caledonian shrimp farming industry every cold season, causing severe epizootic mortalities in grow-out ponds and significant losses. Highly pathogenic strains of Vibrio penaeicida are considered the etiological agent of the disease in Litopenaeus stylirostris. On one hand, studies demonstrated that healthy shrimp may carry V. penaeicida for weeks with a high overall prevalence, regardless of any seasonal pattern or temperature conditions. On the other hand, larvae are free of V. penaeicida and are also resistant to experimental infection. V. penaeicida is frequently detected in incoming water pumped from the bays, which was shown, by a molecular typing study, to be the infectious source. This particular epidemiological pattern highlights the major role of the factors that trigger and aggravate the disease in grow-out ponds, where shrimp populations carry the pathogen all year round. In order to gain a better understanding of “Syndrome 93” epidemiology, quantification of V. penaeicida both in shrimp and the shrimp farm ecosystem is necessary. This article describes the steps in the successful development of a real-time PCR quantification assay of V. penaeicida in shrimp haemolymph, seawater (from ponds or bays) and sediment pore water, including the choice of an accurate extraction technique. The entire detection method; including sample processing, DNA extraction and real-time PCR amplification, can be completed within 4 h

Keywords: Extraction techniques; Mariculture; Quantification; Real-time PCR; Vibrio; Vibriosis
INTRODUCTION

Vibrio species are widely distributed in mariculture facilities throughout the world. Vibriosis is a major disease problem in almost all farmed marine animals, including penaeid shrimp (Lightner and Lewis, 1975; Takahashi et al., 1985; Lightner, 1988; Brock and Lea Master, 1992; Mohney and Lightner, 1994). Our knowledge of the physiopathology of vibriosis in shrimp remains very limited. Indeed, very little knowledge has been gained on this pathology and little is known concerning virulence factors in Vibrio spp that are pathogenic to farmed marine invertebrates. As an alternative to conventional zootechnical practices, sanitation measures could be conceivable to minimize the introduction of pathogenic Vibrio in a non-infected shrimp rearing ecosystem. Still, to meet this objective a rapid, reliable, quantitative and sensitive method for detection of this pathogen would be essential.

New Caledonia is located in the South Pacific between latitudes 19°S and 23°S and has a tropical oceanic climate. Its shrimp industry benefits from an almost virus-free status, IHNV being the only significant virus present, and its domestic stock of Litopenaeus stylirostris being resistant to it (Weppe et al., 1992). From 1993, shrimp growers have had to face a seasonal vibriosis named Syndrome 93 (Goarant et al., 1996; Mermoud et al., 1998) caused by infection with V. penaeicida (Costa et al., 1998; Goarant et al., 1999; Saulnier et al., 2000). Therefore, the shrimp farmers’ association considers the research on vibriosis as the main priority. As a response, the Aquaculture Department of IFREMER (French Institute for Marine Sciences) has developed a specific research program, named DeSanS (possibly translated by Stylirostris Health Challenge). This research program is based on a multi-disciplinary approach; comprising of rearing technology, pond ecosystem studies, shrimp ecophysiology and immunology, nutrition, pathology and genetics. It aims
at gaining a global explanation scheme of the seasonal vibriosis (Harache and Herbland, 2004) as a contribution to strengthen the sustainability of New Caledonian aquaculture.

Conventional laboratory diagnosis usually relies on gross observations, wet mounts, histology and culture. Reference diagnosis includes culture and isolation of the bacteria from the shrimp haemolymph, by using suitable media and subsequent identification. These methods are time-consuming and require several days to obtain confirmatory results. Additionally, lack of culturability has been frequently described in *Vibrios* (Huq and Colwell, 1995; Fischer-Le-Saux et al., 2002), and in some cases these viable but nonculturable cells demonstrated to still remain infectious (Rosenberg and Ben-Haim, 2002; Baffone et al., 2003). Conventional polymerase chain reaction (PCR) method had the potential to make a dramatic impact in diagnosing infectious diseases. Using Arbitrarily Primed PCR, a previous study performed in New Caledonia with local *V. penaeicida* isolates demonstrated that *V. penaeicida* was introduced in the ponds with the renewal seawater pumped from the bays (Goarant et al., 1999). Using PCR detection of *V. penaeicida* (Saulnier et al., 2000), a wide epidemiological survey carried out over more than one year confirmed these findings and demonstrated that healthy carriers were found all year round in grow-out ponds (Goarant et al., 2004). This study evidenced the importance of triggering factors that turn covert infection into septicaemia. However, this PCR approach requires analysis of amplified DNA in an agarose gel, which again is time-consuming and a potential source of carry-over contamination. Additionally, this study did not allow accurate quantification of infection levels in shrimp or concentrations of pathogenic *V. penaeicida* in pumping water. Moreover, PCR inhibitors often impeded the detection of *V. penaeicida* in seawater and detection was not possible in complex marine samples such as sediments. As such, the seasonal dynamics of *V. penaeicida* in New Caledonia remain unknown; underlying the need for an accurate tool allowing the
quantification of *V. penaeicida* in shrimp and ecosystem’s compartments. Recently, the introduction of real-time PCR amplification methods using SYBR Green I as the detection technology, has made detection of bacterial pathogens such as *Vibrio parahaemolyticus* (Blackstone, 2003) and *Vibrio vulnificus* (Panicker, 2004) rapid and cost-effective.

The detection of microbial nucleic acid for the diagnosis of infection is dependent on the successful separation of nucleic acid from material (Read, 2001). In the present study we describe optimization of a real-time PCR assay using the SYBR Green I technology for rapid and sensitive detection and quantification of *V. penaeicida* in biological (shrimp) and environmental (seawater and sediment pore water) samples. Such quantitative information obtained with our real-time PCR assay is important to obtain better knowledge of the implication of *V. penaeicida* in shrimp disease and to apply better zootechnical practices. Oligonucleotide primers (Saulnier et al., 2000) targeted a segment of the *rrs* gene. Various nucleic acids extraction methods were used and compared, including the classic technique of phenol chloroform separation, the release of nucleic acid augmented by digestion with proteinases, and the non-specific adsorption to a matrix of silica particles held within a disposable plastic column.

**MATERIALS AND METHODS**

**Bacterial strains.** AM101 strain was isolated in 1995 from a haemoculture of a moribund shrimp suffering Syndrome 93 and was used as the reference New Caledonian *V. penaeicida* isolate. For DNA extraction, serial dilutions of cultures in marine broth (late exponential phase) were plated in triplicates on Marine Agar 2216E (Difco Laboratories, Detroit, Mich.) allowing estimation of bacterial density. Pure cultures or 10-fold serial dilutions were then used. Suspensions were centrifuged at 15,000 x g for 15 minutes at 4°C and the pellets were washed twice with PBS (final volume up to 200 µl). To assess the
specificity of our real-time assay, we used strains from other *Vibrio* species (*V. penaeicida* KH-1\textsuperscript{T}, *V. alginolyticus*, *V. harveyi*, *V. nigripulchritudo*, *V. aesturianus*, *V. tubiashii*, *V. damsela*, *V. orientalis*, *V. splendidus*, *V. fluvialis* and *V. nereis*). Bacterial strains other than members of the genus *Vibrio* (*Proteus mirabilis*, *Klebsiella pneumoniae*, *Escherichia coli*, *Staphylococcus aureus*, *Streptococcus agalactiae*, *Acinetobacter baumannii*, *Shigella flexneri*, *Salmonella* sp.) were subcultured and the respective DNAs were extracted and purified as described by Brenner et al. (1999).

**Shrimp and haemolymph samples.** Thirty juvenile *Litopenaeus stylirostris* from a grow-out pond (mean weight 8 g.) were used for this study. Twenty were infected with a *V. penaeicida* AM101 suspension as described by Saulnier et al. (2000). Prior to haemolymph sampling, shrimp were rinsed with sterile seawater. Then, haemolymph was withdrawn from the ventral sinus using a sterile tuberculin syringe and needle, immediately placed on ice and used for DNA extraction. Uninfected shrimp (control), moribund and survivors were sampled, in order to get a large range of *V. penaeicida* concentrations in haemolymph specimens.

**Collection of seawater and sediment pore water samples.** Seawater (15 ml) - both from a shrimp grow-out pond and from pumping water - was sampled in a sterile plastic tube. Sediment was sampled in the same pond using a core sampler. Pore water was obtained by low-speed centrifugation of 30 g of sediment (200 x g for 5 minutes at 4°C). Water and pore water samples (200 or 1,200 µl) were then pelleted at 20,000 x g for 15 minutes at 4°C. The supernatant was discarded and the pellet immediately chilled on ice and used as the sample for DNA extraction.

**DNA extraction techniques.** DNAs were extracted and purified by three different methods. (i) After lysis and digestion with a 0.5% sodium dodecyl sulfate (SDS)-0.1 mg.ml\textsuperscript{-1} proteinase K solution (1 to 3 hours incubation at 56°C), bacterial nucleic acids
were extracted by a phenol-chloroform-isoamyl alcohol (25:24:1, vol/vol/vol) mixture as described by Jackson et al. (1991). The DNA was resuspended in 200 µl PCR grade water with 50 µg.ml\(^{-1}\) RNase A (Sigma), incubated 15 min at 37°C and stored at -80°C until use. (ii) Crude shrimp haemolymph (10 µl) or bacterial suspensions (5 µl pure culture or serial dilutions) or environmental samples pellets were mixed with 400 µl of a 5% (wt/vol) Chelex 100 suspension (Sigma Chemical Co., St. Louis, Mo.) prepared in 10 mM Tris buffer pH 8.0. Fifty µl of a 25 µg.ml\(^{-1}\) proteinase K solution was then added and samples were incubated for 4 hours at 56°C, boiled for 15 minutes, placed on ice and stored at -20°C until use. (iii) Suspensions (from bacterial culture or serial dilutions, seawater or sediment pore water) and haemolymph samples (from 10 to 200 µl) were used as templates and extracted with the High Pure™ PCR Template Preparation kit (Roche Diagnostics, New Zealand) as recommended by the manufacturer, following the “whole blood” instructions for this column-based procedure. Eluted DNA was resuspended in 200 µl prewarmed elution buffer and stored at –20°C until use.

**Primers and real time PCR conditions.** Primers VpF (5’-GTGTGAAGTTAATAGCTTCATATC-3’) and VR (5’-CGCATCTGAGTGTCAGTATCT-3’) were used from the \(rrs\) gene as described by Saulnier et al. (2000). The primers (Proligo Singapore Pte Ltd) amplified a 310-bp product from \(V.\) penaeicida. Amplification products were analysed by electrophoresis in 2% NuSieve 3:1 gel agarose (FMC BioProducts, Rockland, ME) with ethidium bromide staining. The LightCycler FastStart DNA Master SYBR Green I kit (Roche Diagnostics, New Zealand) was used as the basis for the reaction mixture, using a 20 µl volume in each reaction capillary. The reaction master mix included a dNTP mix (with dUTP instead of dTTP), SYBR Green I dye, FastStart Taq DNA polymerase (a hot-start enzyme), 4 mM MgCl\(_2\) and 0.5 µM of each primer. After distributing 18 µl aliquots of the master mix
among the capillaries, 2 µl of the DNA was added before the capillaries were capped, centrifuged and placed in the LightCycler sample carousel. A negative control with PCR-grade water rather than template DNA was always used with the samples. Aerosol-barrier pipette tips were used throughout the procedure. The PCR conditions were optimised regarding the annealing temperature, MgCl₂ concentration and sample DNA volume. Amplification conditions involved a pre-incubation at 95°C for 10 min (FastStart Taq DNA polymerase activation) followed by amplification of the target DNA for 50 cycles (95°C for 8 sec, 60°C for 5 sec and 72°C for 13 sec) with a transition rate of 20°C/sec. Melting curve analysis was performed at a linear temperature transition rate of 0.1°C/sec from 65°C to 95°C with continuous fluorescence acquisition. This step was followed by a cooling step at 40°C for 30 sec. The first derivative of the initial melting curve (-dF/dT) was automatically plotted against temperature for improved determination of the melting temperature (Tm). All experiments were repeated at least twice for reproducibility.

RESULTS

Specificity of detection. The specificity of our real-time assay was evaluated by testing the members of the genus *Vibrio*, mentioned above, which covered a broad range of *Vibrio* species and a range of other species covering phylogenetically related species, and representatives of species from other branches. Our real-time PCR assay only amplified the DNA of *V. penaeicida* AM101 and KH¹⁰ reference strains. No amplification was seen with any of the non-*V. penaeicida* or other non-*Vibrio* bacterial species tested in this study. All PCR products of amplified samples were visualized by gel electrophoresis (5 µl of each PCR product on a 1.5% agarose gel containing ethidium bromide) and showed a unique 310-bp band (Fig. 1). Additionally, fluorescence melting curve analysis showed a
specific discriminant melting temperature. Indeed, a mean melting temperature of 87.1°C was obtained for both *V. penaeicida* reference strains (Fig. 2). All negative samples showed no amplification caused by primer-dimer formation (Fig. 2).

**Determination of the optimal extraction technique and quantification of** *V. penaeicida* **by real time PCR.** In order to assess the quality of the extraction process, comparative amplifications were run between DNAs extracted from 10-fold dilutions of a *V. penaeicida* culture. The relative concentration of *Vibrio* DNA recovered from samples by the different extraction methods was measured by determining the PCR cycle at which amplicon specific fluorescence became detectable by crossing a noise band positioned above background values of fluorescence using the LightCycler software (Read, 2001). Representative results with the three extraction methods over a 6-log-unit range of concentration (1 to 10⁵ CFU.mL⁻¹) are shown in Figure 3. The whole extraction and amplification using each method where repeated five times. Briefly, with Chelex preparations or DNAs extracted with High Pure™ PCR Template Preparation Kit, comparable amplification results were obtained. The detection of *V. penaeicida* from pure cultures, using the column-based High Pure™ kit, was possible over a 8-log-unit linear range of concentration (1 to 10⁷ CFU.mL⁻¹) and over a 6-log-unit linear range (1 to 10⁵ CFU.mL⁻¹) using Chelex preparations . The detection limit appeared to be around one single cell per reaction capillary. The correlation between quantitative results (concentrations of *V. penaeicida* cells) was in excellent accordance for both extraction procedures (Chelex, silica matrix), as illustrated in Fig. 3B and 3C, with respective *r* values of 0.9955 and 0.9854. The variation in the extraction (both previous methods) and amplification efficiency for 5 recorded runs (with 5 replicates for each *Vibrio* dilution) was <2% relative standard deviation. At the opposite, when working with phenol-chlororform-isoamyl alcohol extracts, large differences were observed for all replicates of a same
dilution (Fig. 3A). Indeed, compared to Chelex and High Pure™ PCR Template Preparation Kit, for each dilution tested, the mean difference was greater and not acceptable. Therefore, field samples (either haemolymph, pond or bay water, sediment pore water) were only extracted with either the Chelex or High Pure™ techniques.

Real time PCR with field samples. Samples from various compartments of the shrimp farm ecosystem were tested identically. It should be noted that we experienced difficulty obtaining “naturally” infected shrimps, so our panel only contained a limited number of experimentally infected shrimps in laboratory conditions. For shrimp haemolymph specimens, best results were obtained when the initial sample volume was 10 µL, when compared to 50, 100 or 200 µL. Indeed, from 100 µL on, haemolymph clotting appeared to be the major factor inducing low extraction yields. Our samples demonstrated various concentrations of V. penaeicida genomes concentrations, ranging from “not detectable” (apparently healthy shrimp and shrimp surviving 5 days after experimental infection) to 8.4x10⁴ µL⁻¹ (moribund shrimp, after experimental infection). Still, some apparently healthy shrimp and shrimp surviving experimental infection displayed concentrations of up to almost 50 V. penaeicida genomes per µl of heamolymph. Chelex preparations and High Pure™ PCR Template Preparation Kit extracts gave similar results as V. penaeicida genomes per initial sample µL, the largest ratio between the two techniques being 2.1 in a moribund shrimp sample.

For water samples, the Chelex™ preparations frequently gave no amplifications. In contrast, High Pure™ PCR Template Preparation Kit extracts gave positive amplifications; best results being obtained when extracting from the pellet from a 200 µL volume of initial sample when compared to a 1200 µL volume. Concentrations (as V. penaeicida genomes
per initial sample mL) ranging from “not detectable” to 660 were found in pumping water, from 330 to 1830 in shrimp pond water and from 52 to 1210 in sediment pore water.

**Discussion:**

Detailed mechanisms of shrimp vibriosis have until now only received poor research attention, so that little knowledge has been gained on this pathology. This has been mainly due to the fact that the use of antibiotic feeds could often get rid of the disease. Nevertheless, the massive use of antibiotics has led to the selection of antibiotic-resistant bacterial strains (Brown, 1989; Karunasagar *et al*., 1994) and highlighted the inefficiency of such an approach. Furthermore some producing countries, including New Caledonia, have totally banned the use of antibiotics in grow-out ponds (Andrier, 2004) and most importing countries have drastically decreased tolerance on antimicrobial residues. Therefore, research programs aiming at gaining a global understanding of shrimp vibriosis are now being developed (Harache and Herbland, 2004). These findings emphasize the need to set up a sensitive and reliable method that can detect and quantify pathogenic *V. penaeicida* both in shrimp and their surrounding ecosystem. The polymerase chain reaction (PCR) has the potential to make a dramatic impact in diagnosing vibriosis, allowing the detection of many *Vibrio* species whatever their culturability. Although *rrs* has been used for conventional PCR of *V. penaeicida* by other investigators (Saulnier *et al*., 2000; Goarant *et al*., 2004), the method is time-consuming and does not provide quantitative results of *V. penaeicida* infectious levels in shrimp and environmental samples. Therefore, it was necessary to transfer this protocol to a real-time PCR platform. This method has already been used to study the major shrimp viral pathogens (Dhar *et al*., 2001; Dhar *et al*., 2002; Durand *et al*., 2003; Tang *et al*., 2004) and was successfully
applied to quantification of human-pathogenic *Vibrio*, and in some cases in marine invertebrates (Campbell and Wright, 2003).

The detection of microbial pathogens in various biological samples by real-time PCR with SYBR Green I dye has been shown to be rapid, reliable, sensitive and cost-effective. As a first step, we evaluated and established a simple, fast and effective DNA purification method that can be used for detection of *V. penaeicida* in unenriched shrimp haemolymph and water samples; without compromising the quality of DNA and real-time PCR efficiency. To this end, the yield from an extraction method should not be influenced by the initial amount of target nucleic acid in the sample. PCR is prone to inhibition (Tsai and Olson, 1992; Wilson, 1997; Frostegard et al., 1999; Watson and Blackwell, 2000; Dumètre and Dardé, 2003) and marine environmental samples are known to contain substances that can potentially interfere with the amplification process (Audemard et al., 2004; Goarant et al., 2004). Therefore an adequate extraction protocol must be used for nucleic acids extraction and purification. In our evaluation, the classical phenol-chloroform-isoamyl alcohol extraction process did not satisfy these requirements. Indeed, the pipetting of the aqueous supernatant could not be efficiently completed. We observed a decreased sensitivity due to the loss of template DNA during multiple processing steps and the amount of proteins in samples may influence the recovery of nucleic acids. Finally, with such results it was impossible to build a robust standard curve and this method was definitively abandoned. Conversely, the Chelex preparation technique showed that there was a strong correlation between the initial amount of target DNA and real-time PCR Cp values as expected. Successive increases in Cp values with the expected melting temperature (87.1°C) were observed as the concentration of template DNA in the samples decreased. The Chelex protocol allows a single-tube technique without lost of nucleic acids, however, all chemical compounds (including PCR-inhibiting substances) present in
the initial sample are collected with nucleic acids. Using a commercial extraction and
purification kit (High Pure™ PCR Template Preparation kit) we could get positive
amplifications from bay seawater, shrimp pond water and even sediment pore water
samples. All of these samples failed to amplify when using Chelex DNA preparations. The
detection limit was around 1 single V. penaeicida genome per reaction capillary. This is 5
times lower than classical PCR with the same primers which allowed detection of 20
genomes per reaction (in a 10µL extract sample) as described by Saulnier et al., (2000).
This very low detection limit is quite common using real time PCR especially when
dealing with multi-copy gene targets, which is the case for rrs (Moreno et al., 2002).

Interestingly, all V. penaeicida strains isolated to date in New Caledonia
demonstrated high pathogenicity when tested by experimental infection in a shrimp model
(Goarant et al., unpublished data), which is quite different from other aquaculture
pathogenic Vibrio spp. (Liu et al., 1996; Zhang et al., 2001; Toranzo et al., 1983).
Therefore we can assume that the quantification of V. penaeicida in shrimp and marine
samples actually corresponds to the quantification of pathogenic V. penaeicida cells. While
studying our samples, we were also able to confirm these previous results. Regarding the
environmental samples, we confirmed that V. penaeicida is frequently present in water
pumped from the bays, which might therefore be the infectious origin for the grow-out
ponds (Goarant et al., 1999; 2004). We also detected V. penaeicida in shrimp pond
sediment pore water, confirming that inhibitors can frequently alter the detection of
pathogenic Vibrio when using inappropriate nucleic acids extraction method such as
Chelex technique (Goarant et al., 2004). Additionally, we confirmed the presence of
V. penaeicida in apparently healthy shrimp (Goarant et al., 2004).

In this study, we successfully developed a real-time PCR assay with SYBR Green I
dye for the quantification of bacterial pathogen V. penaeicida in both shrimp and
environmental samples. This technology enables PCR to be performed with greatly reduced carry-over contamination risk and with minimal hands-on time. Although melting curve analysis with SYBR Green is sometimes considered less specific compared to the use of fluorescent probes, the need of more expensive probes is not always necessary when conditions of amplification have been correctly optimized (especially primer sequences and concentrations). The specific product melting peaks with no primer-dimer or other non-specific product signal provided evidence that our assay is specific. Moreover, our turnaround time was considerably faster with the new real-time PCR assay. The complete process - including sample processing, extraction of DNA and real-time PCR amplification with quantification - was achieved within 4 hours, making it a single-day assay and a prospective decision tool for shrimp growers, depending on the quantification results. As a research tool, it will allow an accurate survey of \textit{V. penaeicida} dynamics in the shrimp farm ecosystem, including a possible seasonal pattern in the seawater from the bays - water temperature being a major trigger in Syndrome 93 occurrence (Goarant \textit{et al}., 2000). It can also be used to study the dynamics of the pathogen in experimentally infected shrimp, as shown with our preliminary data. In the enzootic context of New Caledonia, it will also be a tool to study the effect on healthy carriage of various parameters, either intrinsic like molt stage, developmental stage (Goarant \textit{et al}., 1998), juvenile age, weight or extrinsic (water temperature, dissolved oxygen, ammonia concentration, feed quality or feed additives, probiotics).

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References:


Figure 1. Example of real-time PCR amplification of strains from various *Vibrio* species and bacterial strains other than members of the genus *Vibrio* demonstrated by agarose gel electrophoresis after ethidium bromide staining. Two ng of template DNA was used in the PCR (50 cycles with primers VpF and VR). Lanes: (1) *V. penaeicida* AM101, (2) *V. penaeicida* KH-1<sup>T</sup>, (3) *V. alginolyticus*, (4) *V. harveyi*, (5) *V. nigripulchritudo*, (6) *V. aesturianus*, (7) *V. tubiashii*, (8) *V. damsela*, (9) *V. orientalis*, (10) *V. splendidus*, (11) *V. fluvialis* (12) *V. nereis*, (13) *Proteus mirabilis*, (14) *Klebsiella pneumoniae*, (15) *Escherichia coli*, (16) *Staphylococcus aureus*, (17) *Streptococcus agalactiae*, (18) *Acinetobacter baumannii*, (19) *Shigella flexneri*, (20) *Salmonella* sp. (PM) Step Ladder 50 bp (Sigma Chemical Co. St. Louis, Mo.) used as a DNA size marker (in base pairs).

Figure 2. (A) Representative results of *V. penaeicida* AM101 and KH-1<sup>T</sup> amplicons detection in channel F1. The fluorescence values versus cycle number are displayed. Two ng of purified DNA (Brenner *et al.*, 1999) were used as positive control. As a negative control (NC), the template DNA was replaced with PCR-grade water. (B) Melting curve analysis after real-time amplification with VpF and VR primers and SYBR Green dye in the LightCycler.

Figure 3. Quantitative results obtained for *Vibrio penaeicida* after extraction by phenol-chlororform-isoamyl alcohol (A), Chelex (B) and Roche High pure PCR preparation kit (C). Cp (crossing point) values versus *Vibrio* density (log<sub>10</sub> CFU.mL<sup>-1</sup>) are displayed. Five replicates were tested with the different dilutions of *Vibrio*. The triangles (open symbols) represent the mean difference between the quantitative results (squares, closed symbols). Error bars indicate the standard deviation. The solid line represents the correlation between the Cp values and *Vibrio* concentrations.
Figure 1
Figure 2

(A) Fluorescence (F1) versus cycle number for V. penaeicida AM101 and V. penaeicida KH-1<sup>T</sup>.

(B) Fluorescence change (d(F1)/dT) versus temperature (°C) for V. penaeicida KH-1<sup>T</sup> and V. penaeicida AM101. NC indicates non-template control.
Figure 3

A

B

C

\[ \log_{10} V.\ penaeicidae\ CFU/mL^{-1} \]