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**Journal of Microbiological Methods**
Volume 67, Issue 1 , October 2006, Pages 27-35
http://dx.doi.org/10.1016/j.mimet.2006.02.013
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**Archimer,** archive institutionnelle de l'Ifremer
http://www.ifremer.fr/docelec/

# 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

48 **INTRODUCTION**

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

60 New Caledonia is located in the South Pacific between latitudes 19°S and 23°S and
61 has a tropical oceanic climate. Its shrimp industry benefits from an almost virus-free status,
62 IHHNV being the only significant virus present, and its domestic stock of Litopenaeus
63 stylirostris being resistant to it (Weppe et al., 1992). From 1993, shrimp growers have had
64 to face a seasonal vibriosis named Syndrome 93 (Goarant et al., 1996 ; Mermoud et al.,
65 1998) caused by infection with V. penaeicida (Costa et al., 1998 ; Goarant et al., 1999 ;
66 Saulnier et al., 2000). Therefore, the shrimp farmers' association considers the research on
67 vibriosis as the main priority. As a response, the Aquaculture Department of IFREMER
68 (French Institute for Marine Sciences) has developed a specific research program, named
69 DeSanS (possibly translated by Stylirostris Health Challenge). This research program is
70 based on a multi-disciplinary approach; comprising of rearing technology, pond ecosystem
71 studies, shrimp ecophysiology and immunology, nutrition, pathology and genetics. It aims

72 at gaining a global explanation scheme of the seasonal vibriosis (Harache and Herbland,
73 2004) as a contribution to strengthen the sustainability of New Caledonian aquaculture.

74 Conventional laboratory diagnosis usually relies on gross observations, wet mounts,
75 histology and culture. Reference diagnosis includes culture and isolation of the bacteria
76 from the shrimp haemolymph, by using suitable media and subsequent identification.
77 These methods are time-consuming and require several days to obtain confirmatory results.
78 Additionally, lack of culturability has been frequently described in Vibrios (Huq and
79 Colwell, 1995; Fischer-Le-Saux et al., 2002), and in some cases these viable but
80 nonculturable cells demonstrated to still remain infectious (Rosenberg and Ben-Haim,
81 2002; Baffone et al., 2003). Conventional polymerase chain reaction (PCR) method had
82 the potential to make a dramatic impact in diagnosing infectious diseases. Using
83 Arbitrarily Primed PCR, a previous study performed in New Caledonia with local V.
84 penaeicida isolates demonstrated that V. penaeicida was introduced in the ponds with the
85 renewal seawater pumped from the bays (Goarant et al., 1999). Using PCR detection of
86 V. penaeicida (Saulnier et al., 2000), a wide epidemiological survey carried out over more
87 than one year confirmed these findings and demonstrated that healthy carriers were found
88 all year round in grow-out ponds (Goarant et al., 2004). This study evidenced the
89 importance of triggering factors that turn covert infection into septicaemia. However, this
90 PCR approach requires analysis of amplified DNA in an agarose gel, which again is time-
91 consuming and a potential source of carry-over contamination. Additionally, this study did
92 not allow accurate quantification of infection levels in shrimp or concentrations of
93 pathogenic V. penaeicida in pumping water. Moreover, PCR inhibitors often impeded the
94 detection of V. penaeicida in seawater and detection was not possible in complex marine
95 samples such as sediments. As such, the seasonal dynamics of V. penaeicida in New
96 Caledonia remain unknown; underlying the need for an accurate tool allowing the

97 quantification of V. penaeicida in shrimp and ecosystem's compartments. Recently, the
98 introduction of real-time PCR amplification methods using SYBR Green I as the detection
99 technology, has made detection of bacterial pathogens such as Vibrio parahaemolyticus
100 (Blackstone, 2003) and Vibrio vulnificus (Panicker, 2004) rapid and cost-effective.

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

113

114 **MATERIALS AND METHODS**

115

116 **Bacterial strains.** AM101 strain was isolated in 1995 from a haemoculture of a
117 moribund shrimp suffering Syndrome 93 and was used as the reference New Caledonian
118 V. penaeicida isolate. For DNA extraction, serial dilutions of cultures in marine broth (late
119 exponential phase) were plated in triplicates on Marine Agar 2216E (Difco Laboratories,
120 Detroit, Mich.) allowing estimation of bacterial density. Pure cultures or 10-fold serial
121 dilutions were then used. Suspensions were centrifuged at 15,000 x *g* for 15 minutes at 4°C
122 and the pellets were washed twice with PBS (final volume up to 200 µl). To assess the

123 specificity of our real-time assay, we used strains from other Vibrio species (V. penaeicida
124 KH-1$^T$, V. alginolyticus, V. harveyi, V. nigripulchritudo, V. aesturianus, V. tubiashii,
125 V. damsela, V. orientalis, V. splendidus, V. fluvialis and V. nereis). Bacterial strains other
126 than members of the genus Vibrio (Proteus mirabilis, Klebsiella pneumoniae, Escherichia
127 coli, Staphylococcus aureus, Streptococcus agalactiae, Acinetobacter baumanni, Shigella
128 flexneri, Salmonella sp.) were subcultured and the respective DNAs were extracted and
129 purified as described by Brenner et al. (1999).

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

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

145 **DNA extraction techniques.** DNAs were extracted and purified by three different
146 methods. (i) After lysis and digestion with a 0.5% sodium dodecyl sulfate (SDS)-0.1
147 mg.ml$^{-1}$ proteinase K solution (1 to 3 hours incubation at 56°C), bacterial nucleic acids

148 were extracted by a phenol-chloroform-isoamyl alcohol (25:24:1, vol/vol/vol) mixture as
149 described by Jackson et al. (1991). The DNA was resuspended in 200 µl PCR grade water
150 with 50 µg.ml$^{-1}$ RNase A (Sigma), incubated 15 min at 37°C and stored at -80°C until use.
151 (ii) Crude shrimp haemolymph (10 µl) or bacterial suspensions (5 µl pure culture or serial
152 dilutions) or environmental samples pellets were mixed with 400 µl of a 5% (wt/vol)
153 Chelex 100 suspension (Sigma Chemical Co., St. Louis, Mo.) prepared in 10 mM Tris
154 buffer pH 8.0. Fifty µl of a 25 µg.ml$^{-1}$ proteinase K solution was then added and samples
155 were incubated for 4 hours at 56°C, boiled for 15 minutes, placed on ice and stored at -
156 20°C until use. (iii) Suspensions (from bacterial culture or serial dilutions, seawater or
157 sediment pore water) and haemolymph samples (from 10 to 200 µl) were used as templates
158 and extracted with the High Pure™ PCR Template Preparation kit (Roche Diagnostics,
159 New Zealand) as recommended by the manufacturer, following the "whole blood"
160 instructions for this column-based procedure. Eluted DNA was resuspended in 200 µl
161 prewarmed elution buffer and stored at –20°C until use.

162 **Primers and real time PCR conditions.** Primers VpF (5'-
163 GTGTGAAGTTAATAGCTTCATATC-3') and VR (5'-
164 CGCATCTGAGTGTCAGTATCT-3') were used from the rrs gene as described by
165 Saulnier et al. (2000). The primers (Proligo Singapore Pte Ltd) amplified a 310-bp product
166 from V. penaeicida. Amplification products were analysed by electrophoresis in 2%
167 NuSieve 3:1 gel agarose (FMC BioProducts, Rockland, ME) with ethidium bromide
168 staining. The LightCycler FastStart DNA Master SYBR Green I kit (Roche Diagnostics,
169 New Zealand) was used as the basis for the reaction mixture, using a 20 µl volume in each
170 reaction capillary. The reaction master mix included a dNTP mix (with dUTP instead of
171 dTTP), SYBR Green I dye, FastStart Taq DNA polymerase (a hot-start enzyme), 4 mM
172 $MgCl_2$ and 0.5 µM of each primer. After distributing 18 µl aliquots of the master mix

173 among the capillaries, 2 µl of the DNA was added before the capillaries were capped,
174 centrifuged and placed in the LightCycler sample carousel. A negative control with PCR-
175 grade water rather than template DNA was always used with the samples. Aerosol-barrier
176 pipette tips were used throughout the procedure. The PCR conditions were optimised
177 regarding the annealing temperature, $MgCl_2$ concentration and sample DNA volume.
178 Amplification conditions involved a pre-incubation at 95°C for 10 min (FastStart Taq
179 DNA polymerase activation) followed by amplification of the target DNA for 50 cycles
180 (95°C for 8 sec, 60°C for 5 sec and 72°C for 13 sec) with a transition rate of 20°C/sec.
181 Melting curve analysis was performed at a linear temperature transition rate of 0.1°C/sec
182 from 65°C to 95°C with continuous fluorescence acquisition. This step was followed by a
183 cooling step at 40°C for 30 sec. The first derivative of the initial melting curve (-dF/dT)
184 was automatically plotted against temperature for improved determination of the melting
185 temperature (Tm). All experiments were repeated at least twice for reproducibility.
186
187 **RESULTS**
188
189 **Specificity of detection.** The specificity of our real-time assay was evaluated by
190 testing the members of the genus *Vibrio*, mentioned above, which covered a broad range of
191 *Vibrio* species and a range of other species covering phylogenetically related species, and
192 representatives of species from other branches. Our real-time PCR assay only amplified the
193 DNA of V. penaeicida AM101 and $KH1^T$ reference strains. No amplification was seen
194 with any of the non-V. penaeicida or other non-Vibrio bacterial species tested in this study.
195 All PCR products of amplified samples were visualized by gel electrophoresis (5 µl of
196 each PCR product on a 1.5% agarose gel containing ethidium bromide) and showed a
197 unique 310-bp band (Fig. 1). Additionally, fluorescence melting curve analysis showed a

198 specific discriminant melting temperature. Indeed, a mean melting temperature of 87.1°C
199 was obtained for both V. penaeicida reference strains (Fig. 2). All negative samples
200 showed no amplification caused by primer-dimer formation (Fig. 2).

201 **Determination of the optimal extraction technique and quantification of**
202 **V. penaeicida by real time PCR.** In order to assess the quality of the extraction process,
203 comparative amplifications were run between DNAs extracted from 10-fold dilutions of a
204 V. penaeicida culture. The relative concentration of Vibrio DNA recovered from samples
205 by the different extraction methods was measured by determining the PCR cycle at which
206 amplicon specific fluorescence became detectable by crossing a noise band positioned
207 above background values of fluorescence using the LightCycler software (Read, 2001).
208 Representative results with the three extraction methods over a 6-log-unit range of
209 concentration (1 to $10^5$ CFU.mL$^{-1}$) are shown in Figure 3. The whole extraction and
210 amplification using each method where repeated five times. Briefly, with Chelex
211 preparations or DNAs extracted with High Pure™ PCR Template Preparation Kit,
212 comparable amplification results were obtained. The detection of V. penaeicida from pure
213 cultures, using the column-based High Pure™ kit, was possible over a 8-log-unit linear
214 range of concentration (1 to $10^7$ CFU.mL$^{-1}$) and over a 6-log-unit linear range (1 to $10^5$
215 CFU.mL$^{-1}$) using Chelex preparations . The detection limit appeared to be around one
216 single cell per reaction capillary. The correlation between quantitative results
217 (concentrations of V. penaeicida cells) was in excellent accordance for both extraction
218 procedures (Chelex, silica matrix), as illustrated in Fig. 3B and 3C, with respective *r* values
219 of 0.9955 and 0.9854. The variation in the extraction (both previous methods) and
220 amplification efficiency for 5 recorded runs (with 5 replicates for each Vibrio dilution) was
221 <2% relative standard deviation. At the opposite, when working with phenol-chlororform-
222 isoamyl alcohol extracts, large differences were observed for all replicates of a same

223 dilution (Fig. 3A). Indeed, compared to Chelex and High Pure™ PCR Template
224 Preparation Kit, for each dilution tested, the mean difference was greater and not
225 acceptable. Therefore, field samples (either haemolymph, pond or bay water, sediment
226 pore water) were only extracted with either the Chelex or High Pure™ techniques.
227
228
229 **Real time PCR with field samples.** Samples from various compartments of the shrimp
230 farm ecosystem were tested identically. It should be noted that we experienced difficulty
231 obtaining "naturally" infected shrimps, so our panel only contained a limited number of
232 experimentally infected shrimps in laboratory conditions. For shrimp haemolymph
233 specimens, best results were obtained when the initial sample volume was 10 µL, when
234 compared to 50, 100 or 200 µL. Indeed, from 100 µL on, haemolymph clotting appeared to
235 be the major factor inducing low extraction yields. Our samples demonstrated various
236 concentrations of V. penaeicida genomes concentrations, ranging from "not detectable"
237 (apparently healthy shrimp and shrimp surviving 5 days after experimental infection) to
238 $8.4x10^4.\mu L^{-1}$ (moribund shrimp, after experimental infection). Still, some apparently
239 healthy shrimp and shrimp surviving experimental infection displayed concentrations of up
240 to almost 50 V. penaeicida genomes per µl of heamolymph. Chelex preparations and High
241 Pure™ PCR Template Preparation Kit extracts gave similar results as V. penaeicida
242 genomes per initial sample µL, the largest ratio between the two techniques being 2.1 in a
243 moribund shrimp sample.
244 For water samples, the Chelex™ preparations frequently gave no amplifications. In
245 contrast, High Pure™ PCR Template Preparation Kit extracts gave positive amplifications;
246 best results being obtained when extracting from the pellet from a 200 µL volume of initial
247 sample when compared to a 1200 µL volume. Concentrations (as V. penaeicida genomes

248 per initial sample mL) ranging from “not detectable” to 660 were found in pumping water,
249 from 330 to 1830 in shrimp pond water and from 52 to 1210 in sediment pore water.
250

251 **Discussion:**

252 Detailed mechanisms of shrimp vibriosis have until now only received poor
253 research attention, so that little knowledge has been gained on this pathology. This has
254 been mainly due to the fact that the use of antibiotic feeds could often get rid of the
255 disease. Nevertheless, the massive use of antibiotics has led to the selection of antibiotic-
256 resistant bacterial strains (Brown, 1989; Karunasagar et al., 1994) and highlighted the
257 inefficiency of such an approach. Furthermore some producing countries, including New
258 Caledonia, have totally banned the use of antibiotics in grow-out ponds (Andrier, 2004)
259 and most importing countries have drastically decreased tolerance on antimicrobial
260 residues. Therefore, research programs aiming at gaining a global understanding of shrimp
261 vibriosis are now being developed (Harache and Herbland, 2004). These findings
262 emphasize the need to set up a sensitive and reliable method that can detect and quantify
263 pathogenic V. penaeicida both in shrimp and their surrounding ecosystem. The polymerase
264 chain reaction (PCR) has the potential to make a dramatic impact in diagnosing vibriosis,
265 allowing the detection of many Vibrio species whatever their culturability. Although rrs
266 has been used for conventional PCR of V. penaeicida by other investigators (Saulnier et
267 al., 2000 ; Goarant et al., 2004), the method is time-consuming and does not provide
268 quantitative results of V. penaeicida infectious levels in shrimp and environmental
269 samples. Therefore, it was necessary to transfer this protocol to a real-time PCR platform.
270 This method has already been used to study the major shrimp viral pathogens (Dhar et al.,
271 2001 ; Dhar et al., 2002 ; Durand et al., 2003 ; Tang et al., 2004) and was successfully

272 applied to quantification of human-pathogenic Vibrio, and in some cases in marine
273 invertebrates (Campbell and Wright, 2003).

274 The detection of microbial pathogens in various biological samples by real-time
275 PCR with SYBR Green I dye has been shown to be rapid, reliable, sensitive and cost-
276 effective. As a first step, we evaluated and established a simple, fast and effective DNA
277 purification method that can be used for detection of V. penaeicida in unenriched shrimp
278 haemolymph and water samples; without compromising the quality of DNA and real-time
279 PCR efficiency. To this end, the yield from an extraction method should not be influenced
280 by the initial amount of target nucleic acid in the sample. PCR is prone to inhibition (Tsai
281 and Olson, 1992 ; Wilson, 1997 ; Frostegard et al., 1999 ; Watson and Blackwell, 2000 ;
282 Dumètre and Dardé, 2003) and marine environmental samples are known to contain
283 substances that can potentially interfere with the amplification process (Audemard et al.,
284 2004 ; Goarant et al., 2004).Therefore an adequate extraction protocol must be used for
285 nucleic acids extraction and purification. In our evaluation, the classical phenol-
286 chloroform-isoamyl alcohol extraction process did not satisfy these requirements. Indeed,
287 the pipetting of the aqueous supernatant could not be efficiently completed. We observed a
288 decreased sensitivity due to the loss of template DNA during multiple processing steps and
289 the amount of proteins in samples may influence the recovery of nucleic acids. Finally,
290 with such results it was impossible to build a robust standard curve and this method was
291 definitively abandoned. Conversely, the Chelex preparation technique showed that there
292 was a strong correlation between the initial amount of target DNA and real-time PCR Cp
293 values as expected. Successive increases in Cp values with the expected melting
294 temperature (87.1°C) were observed as the concentration of template DNA in the samples
295 decreased. The Chelex protocol allows a single-tube technique without lost of nucleic
296 acids, however, all chemical compounds (including PCR-inhibiting substances) present in

297 the initial sample are collected with nucleic acids. Using a commercial extraction and
298 purification kit (High Pure™ PCR Template Preparation kit) we could get positive
299 amplifications from bay seawater, shrimp pond water and even sediment pore water
300 samples. All of these samples failed to amplify when using Chelex DNA preparations. The
301 detection limit was around 1 single V. penaeicida genome per reaction capillary. This is 5
302 times lower than classical PCR with the same primers which allowed detection of 20
303 genomes per reaction (in a 10µL extract sample) as described by Saulnier et al., (2000).
304 This very low detection limit is quite common using real time PCR especially when
305 dealing with multi-copy gene targets, which is the case for rrs (Moreno et al., 2002).

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

320 In this study, we successfully developed a real-time PCR assay with SYBR Green I
321 dye for the quantification of bacterial pathogen V. penaeicida in both shrimp and

322 environmental samples. This technology enables PCR to be performed with greatly
323 reduced carry-over contamination risk and with minimal hands-on time. Although melting
324 curve analysis with SYBR Green is sometimes considered less specific compared to the
325 use of fluorescent probes, the need of more expensive probes is not always necessary when
326 conditions of amplification have been correctly optimized (especially primer sequences and
327 concentrations). The specific product melting peaks with no primer-dimer or other non-
328 specific product signal provided evidence that our assay is specific. Moreover, our
329 turnaround time was considerably faster with the new real-time PCR assay. The complete
330 process - including sample processing, extraction of DNA and real-time PCR amplification
331 with quantification - was achieved within 4 hours, making it a single-day assay and a
332 prospective decision tool for shrimp growers, depending on the quantification results. As a
333 research tool, it will allow an accurate survey of V. penaeicida dynamics in the shrimp
334 farm ecosystem, including a possible seasonal pattern in the seawater from the bays - water
335 temperature being a major trigger in Syndrome 93 occurrence (Goarant et al., 2000). It can
336 also be used to study the dynamics of the pathogen in experimentally infected shrimp, as
337 shown with our preliminary data. In the enzootic context of New Caledonia, it will also be
338 a tool to study the effect on healthy carriage of various parameters, either intrinsic like
339 molt stage, developmental stage (Goarant et al., 1998), juvenile age, weight or extrinsic
340 (water temperature, dissolved oxygen, ammonia concentration, feed quality or feed
341 additives, probiotics).
342

343 **Acknowledgments**

344 This work was partly supported by grants from the South and North Provinces of New
345 Caledonia, IFREMER and the French Ministry of Research. Thanks are due to Dominique

346 Ansquer and Sophie De Decker for technical help during experimental infection of shrimp

347 and DNA extraction. The authors are indebted to J.F. Mackay for editorial contributions.

348

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472

472 **Figure 1**. Example of real-time PCR amplification of strains from various Vibrio species
473 and bacterial strains other than members of the genus Vibrio demonstrated by agarose gel
474 electrophoresis after ethidium bromide staining. Two ng of template DNA was used in the
475 PCR (50 cycles with primers VpF and VR). Lanes : (1) V. penaeicida AM101, (2)
476 V. penaeicida KH-1$^T$, (3) V. alginolyticus, (4) V. harveyi, (5) V. nigripulchritudo, (6)
477 V. aesturianus, (7) V. tubiashii, (8) V. damsela, (9) V. orientalis, (10) V. splendidus, (11)
478 V. fluvialis (12) V. nereis, (13) Proteus mirabilis, (14) Klebsiella pneumoniae, (15)
479 Escherichia coli, (16) Staphylococcus aureus, (17) Streptococcus agalactiae, (18)
480 Acinetobacter baumanni, (19) Shigella flexneri, (20) Salmonella sp. (PM) Step Ladder 50
481 bp (Sigma Chemical Co. St. Louis, Mo.) used as a DNA size marker (in base pairs).
482
483 **Figure 2**. (A) Representative results of V. penaeicida AM101 and KH-1$^T$ amplicons
484 detection in channel F1. The fluorescence values versus cycle number are displayed. Two
485 ng of purified DNA (Brenner et al., 1999) were used as positive control. As a negative
486 control (NC), the template DNA was replaced with PCR-grade water. (B) Melting curve
487 analysis after real-time amplification with VpF and VR primers and SYBR Green dye in
488 the LightCycler.
489
490 **Figure 3**. Quantitative results obtained for Vibrio penaeicida after extraction by phenol-
491 chlororform-isoamyl alcohol (A), Chelex (B) and Roche High pure PCR preparation kit
492 (C). Cp (crossing point) values versus Vibrio density ($\log_{10}$ CFU.mL$^{-1}$) are displayed. Five
493 replicates were tested with the different dilutions of Vibrio. The triangles (open symbols)
494 represent the mean difference between the quantitative results (squares, closed symbols).
495 Error bars indicate the standard deviation. The solid line represents the correlation between
496 the Cp values and Vibrio concentrations.
497

497 Figure 1

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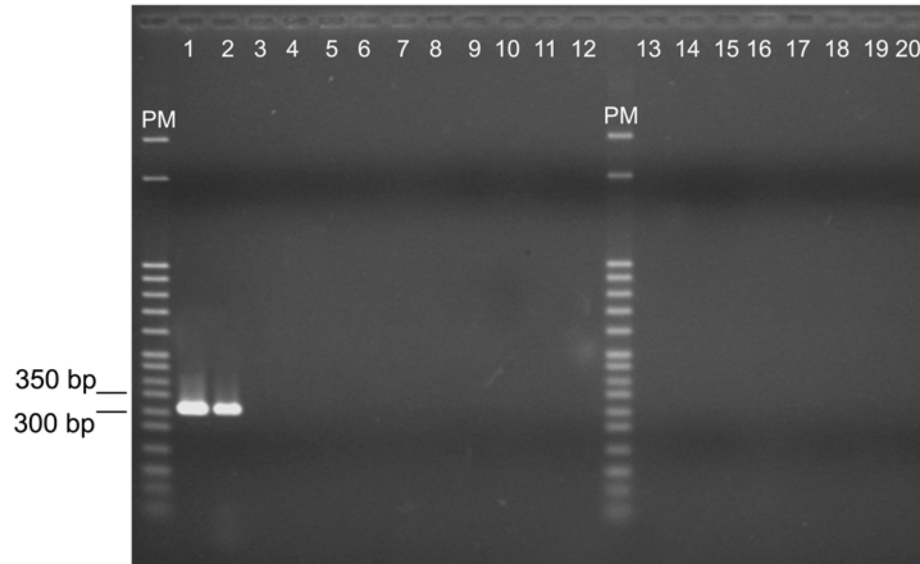


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501 Figure 2

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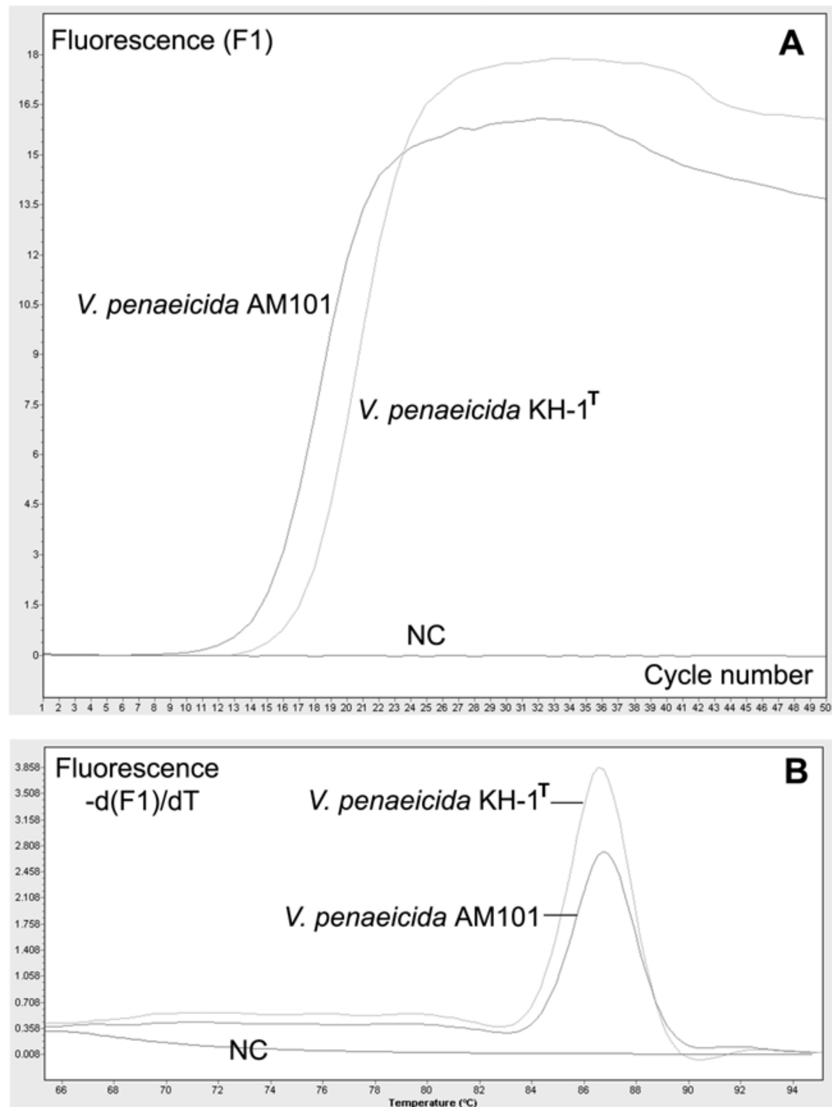


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504 Figure 3

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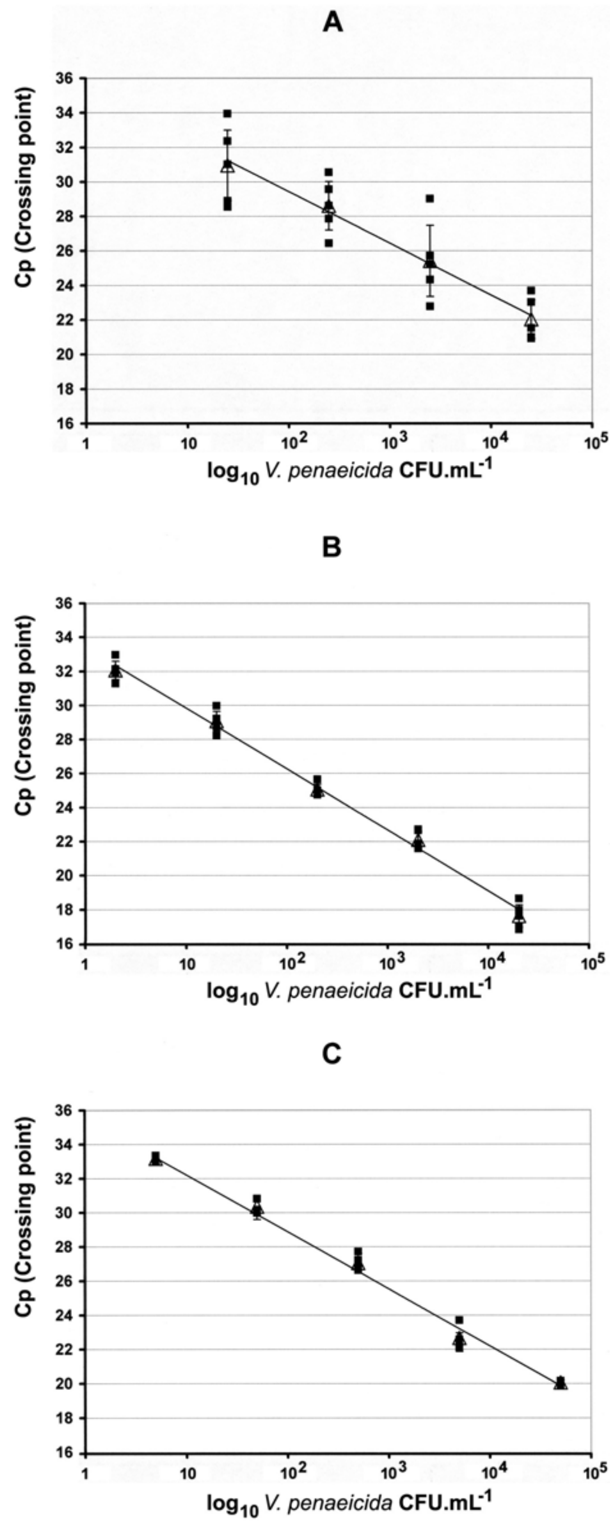


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