Rapid and sensitive PCR detection of *Vibrio penaeicida*, the putative etiological agent of Syndrome 93 in New Caledonia

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ABSTRACT: Experimental infections of *Penaeus* (*Litopenaeus*) *stylirostris* were performed with a *Vibrio penaeicida* strain (AM101) isolated in New Caledonia from Syndrome 93 diseased shrimp. Cumulative mortalities resulting from intramuscular injection or immersion of shrimp in bacterial suspensions demonstrated high virulence for this bacterial strain and suggested that *V. penaeicida* could be the etiological agent of Syndrome 93. The median lethal dose (LD_{50}) for AM101 was 1.3×10^4 CFU (colony forming units) ml^{-1} by immersion and less than 5 CFU $shrimp^{-1}$ by intramuscular challenge, with mortality outbreaks at 48 and 22 h after challenge, respectively. A polymerase chain reaction (PCR) detection assay using a primer set designed from the 16S ribosomal RNA gene of *V. penaeicida* was developed. It gave an expected amplicon of approximately 310 bp in ethidium bromide-stained agarose gels. The specificity of these primers was assessed with different *Vibrio* species. Furthermore, DNA extracted by the Chelex™ method could be used to detect fewer than 20 cultured *Vibrio* cells in seawater or shrimp hemolymph by this assay. It appears to be a reliable screening method for detecting *V. penaeicida* in shrimp and from the aquatic environment.

KEY WORDS: Syndrome 93 · *Vibrio penaeicida* · PCR diagnosis · Shrimp disease · Vibriosis · *Penaeus* (*Litopenaeus*) *stylirostris* · Small subunit rDNA gene

INTRODUCTION

Vibriosis caused by *Vibrio penaeicida* is a disease that affects commercially reared shrimp *Penaeus* (*Marsupenaeus*) *japonicus* in Japan and results in great economic loss (Sano & Fukada 1987, de la Peña et al. 1993, Takahashi et al. 1998). Since 1993, mortality outbreaks have been seasonally observed in semi-intensive *Penaeus* (*Litopenaeus*) *stylirostris* grow out ponds in New Caledonia, principally during winter when water temperatures rapidly drop (Mermoud et al. 1998). Epidemiological and bacteriological studies on this chronic or acute pathology, named 'Syndrome 93', revealed that moribund shrimp always suffered from severe bacterial septicemia mainly due to *Vibrio* species (Costa et al. 1998a, Mermoud et al. 1998). Using

conventional methods for bacterial identification, one group of *Vibrio* strains was found to predominate in moribund shrimp haemolymph. *V. penaeicida* was the main constituent of this group based on ribotypes and DNA percent reassociation in DNA/DNA hybridization assays (Costa et al. 1998a). Nevertheless, less than 70% DNA re-association was found between a *V. penaeicida* reference strain isolated from diseased *P. japonicus* in Japan (Ishimaru et al. 1995) and one isolated from diseased *P. stylirostris* in New Caledonia (Costa et al. 1998a). These strains also exhibited different ribotype profiles, depending on whether the bacterial isolates originated from New Caledonia or Japan. Differences were also observed by molecular typing using an arbitrarily primed polymerase chain reaction (PCR) technique (Goarant et al. 1999).

In this study, we evaluated the pathogenicity of *Vibrio penaeicida* strain AM101 and cloned and se-

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quenced a variable region of its 16S rDNA. Sequence alignment confirmed that it belonged to *V. penaeicida*. Specific primers were designed for a diagnostic assay. We also demonstrated the reliability of the PCR technique as a tool for future epidemiological studies of *V. penaeicida*.

MATERIALS AND METHODS

Vibrio strains and cultivation methods. Four New Caledonian *Vibrio* strains and reference strains belonging to 17 *Vibrio* species were used (Table 1). Bacteria were stored at –80°C and revived on nutrient medium containing 4 g l^{-1} peptone , 1 g l^{-1} yeast extract (Diagnostics Pasteur, Marnes la Coquette, France) and artificial seawater 2.3% wt/vol NaCl, 20 mM KCl, 5 mM MgSO$_4$, 2 mM CaCl$_2$, until they reached the stationary phase by 16 to 20 h incubation at 27°C with agitation. The number of bacteria in culture suspensions was determined by spread plates of 100 µl culture broth on nutrient agar (nutrient medium described above and supplemented with 1.5% agar). After a minimum of 16 h at 27°C, bacterial colonies were counted and results expressed as the number of colony forming units (CFU) per ml of bacterial suspension.

Shrimp infection trials with strain AM101. All our experimental challenges with *Vibrio* isolates were conducted in a controlled area since *V. penaeicida* has not been reported from Tahiti. Shrimp used were healthy juveniles of *Penaeus stylirostris* belonging to the infectious hypodermal and haematopoietic necrosis virus (IHHNV) specific pathogen resistant strain (SPR43) reared in captivity at the Centre Océanologique du Pacifique (COP) for approximately 20 generations. They were acclimated to laboratory conditions in flow-through tanks with no substrate bed for about 1 wk. Several batches, each consisting of 20 shrimp with weights ranging from 10 to 14 g, were kept in 100 l plastic tanks supplied with filtered and aerated water (1 µm) at 24 to 26°C. Juveniles were chosen because postlarval shrimp had been shown to be refractory to experimental infection with AM101 (Goarant et al. 1998).

Experimental infections were performed either by immersion of the shrimp for 2 h in 10 l of seawater containing AM101, or by intramuscular injection between the fifth and sixth abdominal segments of 20 µl of a bacterial suspension containing 250, 2500 or 25 000 CFU ml^{-1} in artificial seawater prepared as indicated above. Control shrimp were similarly injected either with 20 µl of this water or with 20 µl of a suspension of *Vibrio alginolyticus* Z1 strain also isolated from Syndrome 93 diseased shrimp in New Caledonia. After 2 h immersion, the shrimp were rinsed carefully and abun-

Table 1. Strains examined by PCR and specificity of the PCR diagnosis assay using designed primers for *Vibrio penaeicida*. Except for KH1-1^T (= IFO 15660T) isolated from Kuruma prawn in Kagoshima prefecture, Japan, strains of *Vibrio penaeicida* were isolated in New Caledonia from Syndrome 93 diseased *Penaeus stylirostris*. *Vibrio alginolyticus*, Z1 strain, was also isolated in New Caledonia from syndrome 93 diseased *P. stylirostris*. ^{T}Type strain

Vibrio species	Source[a] or strain designations	PCR reaction[b]
V. penaeicida	KH-1^T (= IFO 15660^T)	+
V. penaeicida[c]	AM101	+
V. penaeicida[c]	AM23	+
V. penaeicida	F2	+
V. penaeicida[c]	SF143	+
V. alginolyticus	Z1	–
V. spp.		
(*V. nigripulchritudo?*)	AM100	–
V. alginolyticus	ATCC 17.749^T	–
V. campbelli	CIP 7.067	–
V. carchariae	LMG 7.890	–
V. damsela	CIP 102.761^T	–
V. fluvialis	CIP 103.355^T	–
V. gazogenes	VIB 294^T	–
V. harveyi	LMG 4.044	–
V. holllisae	CIP 101.886^T	–
V. navarensis	CIP 103.381^T	–
V. nereis	LMG 3.895^T	–
V. nigripulchritudo	CIP 103.195	–
V. orientalis	LMG 7.897	–
V. proteolyticus	CIP 102.892	–
V. splendidus	CIP 102.893^T	–
V. tubiashii	CIP 102.760^T	–
V. harveyi/		
V. parahaemolyticus?	98-156/7^d	–
V. parahaemolyticus	69/B3^d	–

[a]ATCC, American Type Culture Collection, Rockville, MD; CIP, Collection de l'Institut Pasteur, Paris, France; IFO, Institute for Fermentation Osaka, Japan; LNG, Belgian Coordinated Collection of Microorganisms, Laboratory voor Microbiologie, Universiteit, Gent

[b]+, only 1 amplification product of 311 bp; –, no amplification products

[c]Strains determined to belong to *V. penaeicida* on the basis of the results of DNA re-association obtained in DNA/DNA hybridization assays using labeled AM23 strain (Costa et al. 1998)

[d]Supplied by Dr Monhey and isolated from diseased shrimp in Madagascar (98-156/7) and Ecuador (90 69/B3)

dantly with filtered seawater and then transferred to tanks (100 l of aerated water). Mortalities were recorded over the following 7 d.

DNA extraction. Except for primer specificity assays where DNA was extracted using a standard SDS, Proteinase K digestion followed by chloroform, phenol extraction (Jackson et al. 1991), genomic DNA was extracted with Chelex™ 100 (Sigma, St. Louis, MO, USA). Briefly, each haemolymph or seawater sample

was centrifuged at 10 000 × g for 10 min, and the supernatant fluid was removed. The pellet was resuspended in 400 µl of a 5% (wt/vol) Chelex™ 100 solution (prepared in 10 mM Tris buffer, pH 8.0, using deionized water). Then 50 µl of Proteinase K (at a final concentration of 25 µg ml⁻¹, Merck, Darmstadt, Germany) was added, and samples were incubated for 4 h at 56°C, boiled at 100°C for 10 min, and immediately put on ice. The supernatant was used directly as template material for PCR, without prior centrifugation and taking care not to pipet the Chelex™ beads.

Cloning and sequencing of a PCR product from *Vibrio penaeicida* AM101 strain 16S rRNA gene. A sense primer, 5'-ccgaattcGGAATATTGCACAATGG-GCGC-3' (VecoF) and an antisense primer, 5'-ggatc-ctcgagGCGCTTTACGCCCAGTAATTCCG-3' (VxhoR) were designed and synthesized (Eurogentec, Seraing, Belgium) in accordance with the procedures for partial 16S rRNA sequence alignments (Kita-Tsukamoto et al. 1993, Genmoto et al. 1996). These primers were flanked at their respective 5' ends with *Eco*RI and *Xho*I cloning restriction sites (shown in the primer sequence as small characters). Their 3'-terminal nucleotide sequences corresponded to 2 highly conserved regions of the 16S rRNA gene among the members of the family *Vibrionaceae*, including the genus *Vibrio*. VecoF and VxhoR primers are located at bases 346 to 366 (Fig. 1) and at bases 541 to 563 in the 16S rRNA gene (GenBank accession number Z83204), respectively, according to the *Escherichia coli* numbering system (Brosius et al. 1981).

PCR amplification was carried out in a 50 µl reaction mixture containing the extracted DNA (25 µl), the reaction buffer (10 mM Tris-HCl, pH 9.0, 50 mM KCl, 2.5 mM MgCl₂, 0.1% Triton X100), 200 µM of each dNTP (Eurogentec), 0.2 µM of each primer and 0.2 U of Gold-star™ DNA polymerase (Eurogentec) in an automatic thermal cycler (MJ Research). The thermal profile involved 35 cycles of denaturation at 94°C for 1 min, annealing at 58°C for 1 min and elongation at 72°C for 1 min, followed by final extension at 72°C for 5 min and holding at 4°C. The resulting PCR product was purified from an agarose electrophoresis gel using a Qiaex® kit (Qiagen, Courtaboeuf, France) and ligated into a *Eco*RI/ *Xho*I-cleaved pBluescript plasmid con-

taining a gene for ampicillin resistance. Competent *Escherichia coli* (DH5α) cells were transformed and successful transformants growing on nutrient agar medium supplemented with ampicillin were then screened by restriction endonuclease analysis. Clones containing rDNA inserts were sequenced and analysed. The determined amplicon sequence was deposited in the GenBank data base under accession number AJ249719.

PCR for diagnostic assays. A sense primer 5'-GTGT-GAAGTTAATAGCTTCATATC-3' (VpF) was selected from a highly variable region of the 16S rRNA gene (Kita-Tsukamoto et al. 1993) after alignment of the AM101 rDNA sequence with other known rDNA sequences from *Vibrio* species at GenBank. By contrast, the reverse primer 5'-CGCATCTGAGTGTCAG-TATCT-3' (VR) was chosen from a conserved region of the 16S rRNA gene found in 17 *Vibrio* species (Kita-

Fig. 1. Partial 16S rRNA sequences of *Escherichia coli* sequence aligned with *Vibro penaeicida* (Genmoto et al. 1996) and cloned *V. penaeicida* strain AM101. Primers for PCR are localised according to *E. coli* numbering. Variable regions of 16S rRDNA of *Vibrionaceae* and specific nucleotides for main groups of *Vibrio* species (Kita-Tsukamoto et al. 1993) are indicated by a box and underlining, respectively. *: non-complementary bases between the primers and the *E. coli* sequences. Nucleotides: N = A,C,G, or T ; Y = pyrimidine (C or T)

Tsukamoto et al. 1993). VpF and VR primers were synthesized by Eurogentec. They were located at bases 440 to 463 and at bases 730 to 750, respectively, in the 16S rRNA gene sequence (GenBank accession number Z83204) from *Escherichia coli* (Fig. 1). PCR reaction mixtures were identical to those described above, except that the reaction was performed with 0.1 U of Goldstar™ DNA polymerase and 10 µl of Chelex™-extracted DNA, in a final volume of 20 µl. The PCR conditions used were 1 min at 94°C for initial denaturation of template DNA and 35 cycles each consisting of 30 s at 94°C (denaturation), 30 s at the selected annealing temperature, 30 s at 72°C (extension), followed by a final round of extension for 5 min at 72°C using the same automated DNA thermal cycler as mentioned before.

Positive controls used for each PCR assay consisted of AM101 DNA extract while negative controls consisted of deionized water. Amplification products were run at a constant voltage of 5 V cm^{-1} on 1 to 2% (wt/vol) agarose gels with TBE electrophoresis buffer (45 mM Tris, 45 mM Borate, 1 mM EDTA). They were stained with 0.5 µg ml^{-1} ethidium bromide and visualized by UV transillumination.

Sensitivity assays. Haemolymph from 20 healthy *Penaeus stylirostris* was withdrawn from the ventral sinus with a 1 ml sterile syringe containing an equal volume of anticoagulant solution (trisodium citrate 30 mM, NaCl 338 mM, glucose 115 mM, EDTA 10 mM, pH 7) and pooled. An AM101 culture of known cell concentration in Zobell medium was serially diluted 10-fold in 0.5 ml aliquots of both haemolymph and seawater, and the CFU ml^{-1} of each sample was verified by the plate count method. Ten or 200 µl of these samples were then taken for DNA extraction. Prior to DNA extraction, the 200 µl samples were centrifuged at 10 000 × *g* for 10 min, the supernatant poured off and the pellet resuspended in 450 µl of Chelex™-Proteinase K solution as already described. The 10 µl samples were extracted directly with 450 µl of Chelex™-Proteinase K solution (i.e. without prior centrifugation).

RESULTS

In shrimp challenged with strain AM101, the median lethal dose (LD$_{50}$) was 1.3×10^4 CFU ml^{-1} by immersion and less than 5 CFU shrimp^{-1} by intramuscular injection, with mortality onset after challenge at 48 and 22 h (Fig. 2b), respectively. No significant mortalities were observed in controls injected with artificial seawater or *Vibrio alginolyticus* Z1. In addition, pure cultures of bacteria were obtained from 20 diseased shrimp, in most cases from the haemolymph spread on agar plates. In other cases, the dominant bacteria were con-
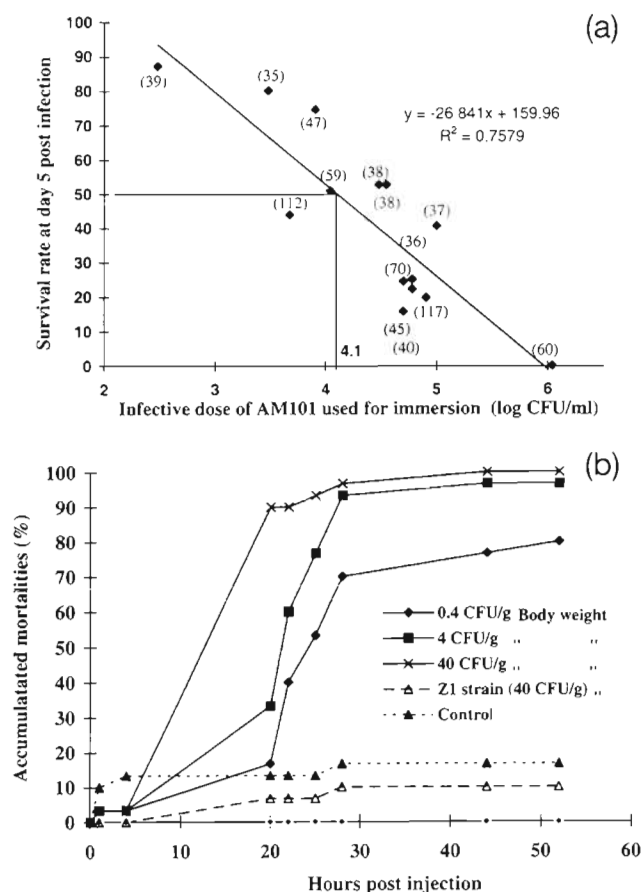

Fig. 2. Mortality data from pathogenicity experiments with AM101 strain. (a) Cumulative mortalities (in %) obtained at Day 5 following 2 h immersion in seawater containing the final concentrations of bacteria indicated. Data were compiled from a series of thirteen 2 h immersion challenges. The number of *Penaeus stylirostris* taken for each challenge is given in brackets. (b) Cumulative mortalities obtained at various hours after intramuscular injection of bacterial suspensions. Each treatment comprised 30 shrimp

firmed as *V. penaeicida* by the PCR assay described herein. PCR identified bacterial isolates from these shrimp succeeded in reproducing morbidities and mortalities by intramuscular injection or immersion challenges, and thus Koch's postulates were fulfilled. In contrast to immersion challenge, there was no relationship between dose and level of mortality using intramuscular injection. More than 50% cumulative mortality was observed within 2 d even with a very low number of injected bacteria, indicating high virulence (Fig. 2). However, results did show some delay in mortality onset when low numbers of bacterial cells were injected. This could correspond to a dose-dependent swiftness in spread of septicemia. Onset of mortality was between 4 and 20 h post-injection using 40 CFU g^{-1} and between 20 and 22 h using 0.4 CFU g^{-1}.
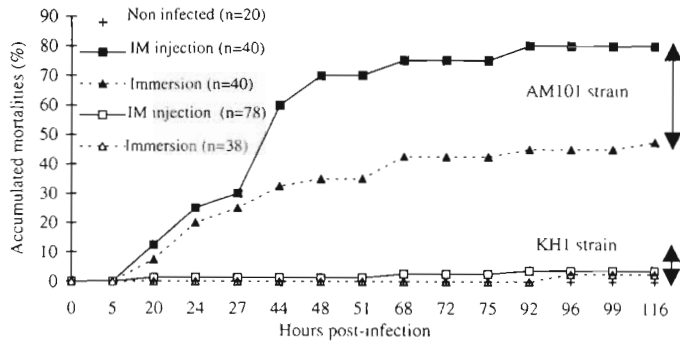
Fig. 3. Virulence of AM101 and KH1 strains in experimental infections. *Penaeus stylirostris* weighing approximately 8 g were infected with AM101 or KH1 strains either by 2 h immersion challenge (doses = 3.5×10^4 CFU ml^{-1} and 4×10^4 CFU ml^{-1}, respectively) or by intramuscular (IM) injection (dose = 3 CFU g^{-1} body weight and 5 CFU g^{-1} body weight, respectively). The number of shrimp for each challenge is given in brackets

Most experimentally infected shrimp exhibited gross signs of classical bacteremia (Lightner & Lewis 1975). Haemolymph samples collected from 30 eye-stalk tagged shrimp infected by immersion showed positive results by PCR 16 h post exposure, before the appearance of gross signs of disease and death. Thus, asymptomatic carriers could be detected by this technique. It is noteworthy that the Japanese type strain KH-1^T iso-lated from *Penaeus japonicus* did not exhibit any noticeable pathogenic effects on *P. stylirostris* (gross signs or mortalities), whatever the route of infection (Fig. 3). This constrasted with *Vibrio penaeicida* isolates from New Caledonia and particularly strain AM101.

Amplification of AM101 DNA extract with VecoR and VxhoR primers resulted in an expected 230 bp single PCR product (Fig. 1). The nucleotide sequence of this 16S rRNA gene fragment was found to be very close, but not identical to that for *Vibrio penaeicida* (Genmoto et al. 1996) by sequence alignment, particularly in the highly variable region (440 to 463 bp, Fig. 1). This provided additional evidence that AM101 isolated in New Caledonia belonged to *V. penaeicida*.

To develop an optimal diagnostic assay, 2 additional primers (VpF) and (VR) (see 'Materials and methods') were tested (Fig. 1). PCR amplification with these primers at a 62°C annealing temperature yielded a single, 310 bp DNA fragment (expected length). Under these conditions, it was the only product obtained with DNA extracted from *Vibrio penaeicida* strains originating from either New Caledonia or Japan (Table 1). No amplification products were observed with the other *Vibrio* species shown in Table 1.
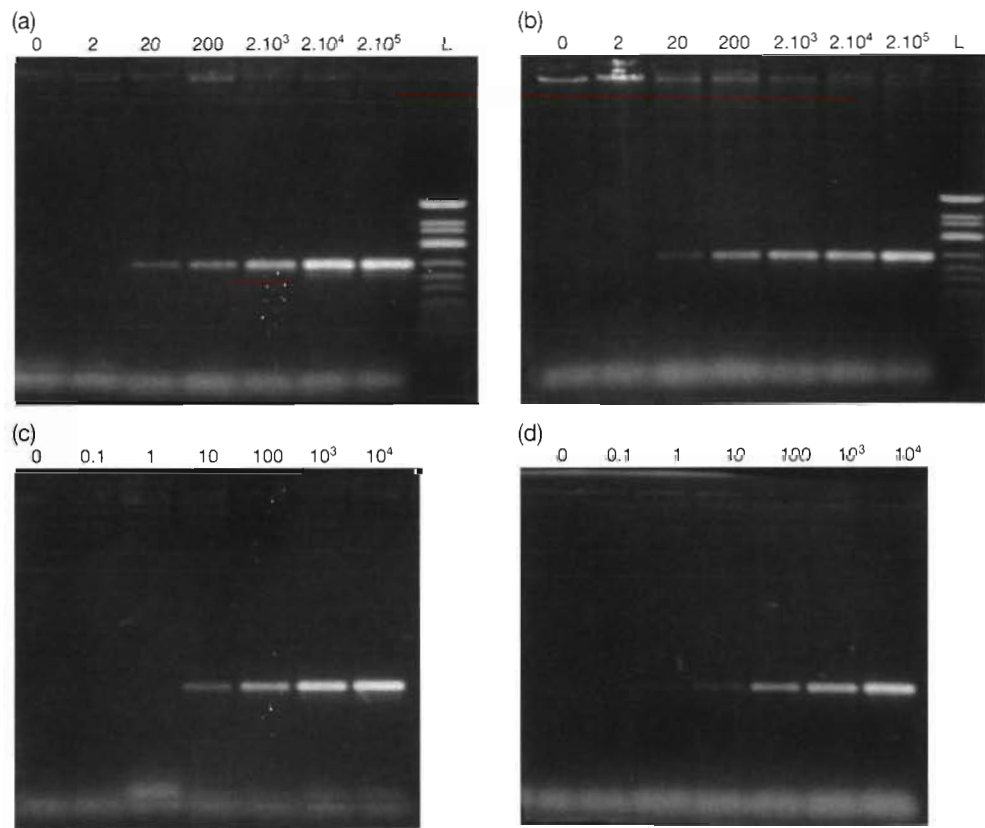

Fig. 4. Agarose gel (1 %) electrophoretic analysis of PCR-amplified products obtained using VpF and VR primers with various concentrations of pure AM101 (a) diluted in seawater or (b) in hemolymph from healthy *Penaeus stylirostris*. Bacterial DNA was Chelex™-extracted either (c,d) without sample centrifugation for 10 µl samples or (a,b) after centrifugation at 10000 × *g* for 200 µl samples. Lanes: L, ladder size markers from ΦX174 plasmid digested with *Hin*fI enzyme (Eurogentec)

In PCR sensitivity tests using serial 10-fold dilutions of AM101 either in seawater or in haemolymph, the detection limit was about 20 *Vibrio penaeicida* genome equivalents as determined by the plate count method (Fig. 4). It is important to note that large haemolymph samples must be centrifuged before DNA extraction. Without centrifugation, no more than 10 μl of haemolymph could be efficiently amplified by PCR using the Chelex™ method. This suggested the presence of some PCR inhibitory compounds in haemolymph. Thus, we recommend that approximately 200 μl be centrifuged to remove plasma and to increase sensitivity of the PCR method. In this way positive results were obtained with dilutions corresponding to 100 *V. penaeicida* CFU per ml of haemolymph (Fig. 4).

DISCUSSION

Experimental shrimp infection by immersion using AM101 allowed reproducible tests with a high correlation between dose and mortality. This constitutes an interesting model for studying the shrimp response to non-invasive infection. Reproducibility was successfully obtained using careful storage of AM101 strain at −80°C, standardized growing techniques in appropriate media and standardized infection schedules in a controlled environment.

The percentage of DNA/DNA reassociation between the New Caledonian isolate (AM23 strain) and the Japanese *Vibrio penaeicida* reference strain (KH1 strain) was found to be approximately 60% (Costa et al. 1998a). Based on the phylogenetic definition of species by Wayne et al. (1987), this would disqualify the 2 strains from being considered the same species. Nevertheless, the level of sequence identity in the variable region of the SSU rRNA gene between the New Caledonian AM101 and the *V. penaeicida* reference strain isolated from Japan indicates that AM101 strain does belong to *V. penaeicida*. Based on the PCR results, other highly pathogenic bacterial isolates from New Caledonia also belong to *V. penaeicida* (Table 1).

The high virulence of the bacterial strains isolated in New Caledonia from Syndrome 93 diseased *Penaeus stylirostris* was demonstrated by experimental infections using 2 different routes of infection and water temperatures not stressful for shrimp. By intramuscular injection, the virulence of New Caledonian *Vibrio penaeicida* isolates on *P. stylirostris* was found to be higher than the Japanese *V. penaeicida* isolates tested by de la Peña et al. (1993) with *P. japonicus*. Our LD$_{50}$ values were lower than 5 CFU shrimp^{-1} (10 to 14 g) while those of de la Peña et al. (1993) were 1×10^2 to 10^3 CFU shrimp^{-1} (13 to 22 g). These results strongly suggest that *V. penaeicida* is a specific rather than

opportunistic agent in contrast to many other *Vibrio* species which produce disease only in stressed shrimp (Lightner 1988). Based on our results, we consider this highly pathogenic *V. penaeicida* strain as the main etiological agent of Syndrome 93, especially since studies on viral etiology have shown little success to date (Costa et al. 1998b).

Several reports have pointed out the low reproducibility and instability of some phenotypic markers commonly used for bacterial identification because of their sensitivity to experimental culture conditions (Hovik Hansen & Sorheim 1991). Furthermore, marker variability is sometimes insufficient to allow differentiation between related bacteria species, and characterization systems for marine bacteria identification are often laborious and time consuming. Therefore, diagnostic identification based on DNA fragment detection is an important alternative to classical phenotypical studies. In our study, this method was rapid, highly sensitive, specific and able to detect viable, but not necessarily culturable bacteria (Morgan et al. 1993). These techniques have been extensively developed for a wide range of pathogenic bacteria, especially those considered to be human health hazards (Wright et al. 1993, Leon et al. 1994, Martinez-Picado et al. 1994, Toyama et al. 1994, Arias et al. 1995, Miyata et al. 1996, Venkateswaran et al. 1998).

Recently, Genmoto et al. (1996) described a diagnostic procedure for the identification of *Vibrio penaeicida* in bacterial cultures isolated from diseased *Penaeus japonicus*. They recommended a reverse transcriptase step before amplification of the partial SSU rDNA sequence of *V. penaeicida* by PCR, in order to increase the sensitivity of the assay. Our results, using the same sense but different antisense primer, indicate that approximately 20 bacterial genome equivalents in seawater or haemolymph samples are sufficient for visual detection of the expected PCR product in agarose gels. Furthermore, specificity of the test was demonstrated using DNA extracts from pure reference cultures of other *Vibrio* species which did not cross-react. The diagnostic test is easy to perform on very large numbers of samples since DNA is much more stable than RNA, and the procedure for DNA extraction by Chelex™ is very fast. Moreover, this Chelex™ method enables DNA extractions directly in the field, and only requires previous preparation of the extraction mixture. If centrifugation cannot be performed in the field, an alternative DNA extraction with smaller amounts of haemolymph or seawater is possible, although this would decrease the sensitivity of the test by a factor of 20.

In conclusion, a sensitive, specific and reliable diagnostic method has been established for detection of *Vibrio penaeicida* both in seawater and in haemolymph of infected shrimp. This method could be of great value

for monitoring the presence of this agent in the marine environment and for correlating environmental factors affecting spatial and temporal distributions of the infectious disease it causes. Finally, rapid identification of *V. penaeicida* (within 12 h after sampling) allows time for intervention to prevent the spread of infections.

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