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# Pathotyping of *Vibrio* Isolates by Multiplex PCR Reveals a Risk of Virulent Strain Spreading in New Caledonian Shrimp Farms

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## Abstract:

Two recurring syndromes threaten the viability of the shrimp industry in New Caledonia, which represents the second largest export business. The "Syndrome 93" is a cold season disease due to *Vibrio penaeicida* affecting all shrimp farms, while the "Summer Syndrome" is a geographically restricted vibriosis caused by a virulent lineage of *Vibrio nigripulchritudo*. Microbiological procedures for diagnosis of these diseases are time-consuming and do not have the ability to discriminate the range of virulence potentials of *V. nigripulchritudo*. In this study, we developed a multiplex PCR method to simultaneously detect these two bacterial species and allow for pathotype discrimination. The detection limits of this assay, that includes an internal amplification control to eliminate any false-negative results, were determined at 10 pg purified DNA and 200 cfu/ml. After confirming the effectiveness of our method using experimentally infected animals, its accuracy was compared to standard biochemical methods during a field survey using 94 samples collected over 3 years from shrimp farms encountering mortality events. The multiplex PCR showed very high specificity for the detection of *V. penaeicida* and *V. nigripulchritudo* (inclusivity and exclusivity 100%) and allowed us to detect the spreading of highly pathogenic isolates of *V. nigripulchritudo* to a farm adjoining the "Summer Syndrome area." This assay represents a simple, rapid, and cost-effective diagnostic tool for implementing timely risk management decisions but also understanding the seasonal and geographical distribution of these pathogens.

**Keywords:** multiplex PCR, Internal Amplification Control (IAC), *Vibrio penaeicida*, *Vibrio nigripulchritudo*, shrimp, *Litopenaeus stylirostris*, diagnosis, virulence, syndrome

## 1. Introduction

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Crustaceans represent one of the most economically important global aquaculture sectors, worth in excess of \$15.7 billion USD in 2007. However, the epizootic spread of a wide variety of pathogens, including viruses, bacteria, fungi and protozoa, has led to huge economic losses in the shrimp farming industry, threatening its economic viability and long-term sustainability in some regions of the world. In New Caledonia (South Pacific), the shrimp culture industry is based upon the Pacific blue shrimp *Litopenaeus stylirostris* and is regularly affected by two seasonal vibrioses responsible for acute mortality outbreaks in specific environmental conditions. The first one, named 'Syndrome 93', mainly occurs during the southern winter (from mid-May to mid-September) and is caused by *Vibrio penaeicida* [8, 24]. The second vibriosis, due to *V. nigripulchritudo*, occurs during the warm season (from mid-November to mid-April). This disease, also called 'Summer Syndrome', is geographically restricted, affecting 3 out of the 17 farms of the island, and can wipe out as much as 80% of a pond around day 55-60 of the 120-day grow-out period [6, 19, 10]. Today, these recurring epizootics threaten the sustainability of this industry, which represents the second largest export business in New Caledonia.

Solutions to control the occurrence and spread of bacterial diseases in marine invertebrates are scarce. The use of antibiotics has been banned in New Caledonian farming procedures, since it may contribute to the development of antibiotic-resistant pathogens and to the dissemination of resistance determinants between aquatic and anthropogenic sources [23, 5]. In addition, whilst there is mounting evidence for specific immune memory in crustaceans, including shrimp [15], it is not yet clear whether any memory to these *Vibrio spp* is elicited, preventing the use of vaccination as a strategy to reduce bacterial disease impact. In light of these limitations, an efficient control of bacterial pathogens relies on the development of rapid and discriminative tests to minimize the introduction and spread of pathogenic agents in non-infected shrimp rearing systems. Molecular-based detection methods, primarily PCR and real-time PCR, have been previously described to detect and quantify *V. nigripulchritudo* and *V. penaeicida* isolates from various environmental and clinical sample types [24, 7, 9]. However, some complications linked to these assays remain unresolved. First, these two bacterial species can co-exist in a shrimp farm as well as in animals. Since these methods target single species, two PCRs are needed for simultaneous detection of these pathogenic agents, rendering the process costly and time-consuming. The 'Summer Syndrome' is caused by a virulent lineage of *V. nigripulchritudo* [6, 10]. Experimental challenges have shown that strains belonging to this lineage exhibit different virulence patterns from moderately pathogenic (20–80% mortality; MP) to highly pathogenic (80–100% mortality; HP). Non-pathogenic isolates of *V. nigripulchritudo* (0–20% mortality; NP) are genetically more diverse. Although existing tools allow the specific identification of strains from this lineage, they are not able to predict their virulence, mostly because of a lack of knowledge regarding the molecular determinants of *V. nigripulchritudo* pathogenesis. However, the recent sequencing of the complete genome of the *V. nigripulchritudo* HP strain SFn1 revealed the presence of two plasmids of 11.7 (pB1067) and 247.2 kb (pA1066), respectively [22, 18]. The distribution and polymorphism of these plasmids, which are absent from NP isolates, strongly suggest that they may be driving forces, as well as markers, for the emergence of the HP phenotype in this bacterial species.

The objective of the present study was to establish a multiplex PCR method containing a chimerical internal amplification control (IAC) to enable the simultaneous detection and typing of *V. penaeicida* and *V. nigripulchritudo* strains. The robustness and applicability of this assay was subsequently determined using field samples collected over 3 years from several shrimp farms facing mortality outbreaks.

## 2. Methods

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### 2.1 Bacterial strains, media and DNA preparation

Strains used for validation of the multiplex PCR are presented in Table 1 and have been previously described [8, 10]. Type strains of different *Vibrio* species were provided by the Centre de Ressources Biologiques (CRB, La Tremblade, France), other bacterial species were obtained from the Pasteur Institute of New Caledonia (IPNC, Nouméa, New Caledonia). *Vibrio* were grown in Marine Broth (MB) or Marine Agar (MA) at 28°C, other bacterial species being cultured in Luria–Bertani (LB) at 37°C. Template DNA was purified from bacteria grown in broth culture with the High Pure™ PCR Template Preparation kit (Roche Diagnostics, New Zealand) following manufacturer's instructions for "isolation of nucleic acids from bacteria". In the case of hemolymph samples or seawater suspensions, DNA was extracted following the "whole blood" instructions, except that each sample was incubated with 5 µl lysozyme (10 mg.ml<sup>-1</sup> in 10 mM Tris-HCl, pH 8.0) for 1 h at 37°C before carrying out the procedure.

### 2.2. Selection of target genes and oligonucleotide primer design

Two genes, *gyrB* and *gapA*, were selected for differential detection of *V. nigripulchritudo* and *V. penaeicida* species, respectively. To design species-specific primers, *gyrB* and *gapA* gene sequences of *V. penaeicida* and *V. nigripulchritudo* along with 28 published sequences of other vibrios were downloaded from GenBank or retrieved from previously published work [10]. Sequences were aligned using the BioEdit software to identify species-specific conserved regions. Primers for *V. penaeicida gapA* gene were designed using the Primer 3 software (<http://frodo.wi.mit.edu/primer3/>), while primers for *V. nigripulchritudo gyrB* gene were deduced from previously designed oligonucleotides with some modifications [9] (Table 2). To differentiate MP from HP strains within the 'Summer Syndrome' lineage of *V. nigripulchritudo*, we used previously designed primers (namely pSFn1-6020 and pSFn1-6974) [27]. These primers target a 954-bp fragment of plasmid pB1067 evidenced only in the HP isolates [22]. Finally, to distinguish MP from NP isolates, we designed a primer pair targeting the *rtxA* gene of plasmid pA1066 from *V. nigripulchritudo*.

### 2.3. Construction of a competitive internal amplification control (IAC)

To identify false-negative results, an IAC was constructed in competition with the primers targeting the *rtxA* gene of *V. nigripulchritudo* using plasmid pUC19 and the procedure of Abdulmawjood et al. [1]. Briefly, the IAC primer set had 5'overhanging ends identical to the *rtxA* gene primer and 3'ends complementary to the pUC19 sequence (Table 2). These primers generate a 376-bp product, including the flanking regions of the primer set. Following PCR amplification, the product was purified using the QIAquick Gel Extraction kit (QIAGEN) and titrated using a NanoDrop<sup>ND</sup> 1000 Spectrophotometer (NanoDrop Technologies). The IAC copy number was calculated as the amount of template  $\times N_A$  / length of template  $\times 1.10^9 \times Mbp$ , where  $N_A$  is Avogadro's number ( $6.022 \times 10^{23} \text{ mol}^{-1}$ ) and  $Mbp$ , the mean molar mass of a base pair ( $650 \text{ g mol}^{-1}$ ).

### 2.4. PCR protocol

Before optimization in a multiplex PCR, each primer pair was tested individually using DNA extracts of either *V. nigripulchritudo* SFn1 (representative HP isolate) or *V. penaeicida* AM101 (reference New Caledonian isolate) as templates. PCR was carried out on a GeneAmp<sup>R</sup> PCR system 9700 thermocycler (Applied Biosystems) or on a MJ<sup>TM</sup> Mini thermal cycler (BioRad) in a 20 µl volume containing genomic DNA, 2 µl of 10 × PCR buffer, 200 µM

dNTP and 1 U of HotStartTaq DNA polymerase. Two parameters were optimized: the annealing temperature and the primer concentration (ranging from 0.2 to 0.8  $\mu\text{M}$  in 0.2  $\mu\text{M}$  increments). The PCR program contained the following steps: initial denaturation of 15 min at 95°C followed by 35 cycles of denaturation at 95°C for 30 s, primer annealing at 50-70°C for 30 s, extension at 72°C for 30 s, and a final extension at 72°C for 5 min. PCR products were subjected to electrophoresis on 1.5% agarose gels containing GelRed™ DNA stain. To confirm their identity, representative amplicons for each target gene were gel-purified and sequenced by a commercial facility (GATC Biotech AG, Konstanz, Germany). Before including the IAC to each reaction, 5-fold dilutions of the IAC template (ranging from  $2.8 \times 10^8$  to 28 copies) were tested in a conventional PCR using *rtxA* gene primer (*rtxA* F and *rtxA* R) to determine the lowest IAC DNA concentration giving a reliable PCR band visible by gel electrophoresis.

## 2.5. Specificity and sensitivity assays

The specificity of the 4 primer sets was assessed together by a multiplex PCR. Reactions were carried out following the previously optimized conditions, using 2  $\mu\text{l}$  of purified DNA and 284 copies of IAC. The detection limits of the assay were determined on DNA extracted from different sample types: shrimp hemolymph, artificial seawater and broth cultures. To this end, exponentially grown *V. nigripulchritudo* SFn1 or *V. penaeicida* AM101 cultures were subjected to 10-fold serial dilutions in 1 ml of artificial seawater or hemolymph, collected as previously described [18]. Viable plate counts were performed in duplicates on MA to determine the cfu/ml of each sample, while DNA extractions were carried out from 200  $\mu\text{l}$  aliquots as described above. The extracted DNA from each sample was used for multiplex PCR amplification, all samples being analyzed in duplicates. In addition, the efficiency of the assay was checked using purified genomic DNA ( $\text{OD}_{260/280} \geq 1.7$ ) Template DNA was 10-fold diluted in water from 10 ng to 0.1 pg, and aliquots from each of the diluted samples were used for multiplex PCR amplifications.

## 2.6. Detection of *V. nigripulchritudo* and *V. penaeicida* in experimentally infected shrimp

Experimental infections were performed by immersion of the shrimp in aerated seawater containing  $10^5$  cfu/ml of the considered *Vibrio* strain, as previously described [18]. Three replicate tanks were used for each treatment (i.e. control, experimentally infected with *V. nigripulchritudo* SFn1 and experimentally infected with *V. penaeicida* AM101). 24 hours after infection, 2 shrimp from each tank were randomly sampled. Hemolymph collection, DNA extraction and plate counts were performed as described above.

## 2.7. Application of the method to field samples

From January 2008 to December 2010, shrimp were sampled during mortality outbreaks occurring in 10 different shrimp farms located on the southwest coast of New Caledonia (Figure 3). This survey was conducted in the context of a health surveillance and monitoring program set up by Ifremer and the New Caledonian government veterinary laboratory. Shrimp were collected from each *V. nigripulchritudo* cells were identified as black colonies in the presence of glycerol [2]. Obtained data were interpreted according to the Bergey's manual of determinative bacteriology (9th ed.). In parallel, these isolates were also analyzed by the multiplex PCR method. When isolates belonging to the *V. nigripulchritudo* species were identified as HP by the assay, their pathotype was confirmed by injecting shrimp with bacterial culture supernatants. Details of supernatant preparation and injection have been previously described [18]. Finally, when isolate identification showed discordant results

between the two methods, 16S rRNA gene amplification was performed by PCR using eubacterial primers 1080.r (5'-GGG ACT TAA CCC AAC ATC T-3') and 370.f (5'-GGC AGC AGT GGG GAA TAT TG-3'). Then ponds by castnet operation, brought to the laboratory in an iced box and immediately processed for bacteriological analyses. One drop of hemolymph was aseptically withdrawn from the shrimp ventral sinus using a 1-ml plastic syringe fitted with a 25-gauge needle and plated on to MA and MA supplemented with 2% glycerol (w/v). Following incubation at 28°C for 48 h, presumptive identification of bacterial isolates to the species level was done by a series of phenotypic tests which included cell morphology and motility, oxidase activity and API 20E (BioMérieux, Marcy L'Etoile, France). Putative

### 3. Results

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#### 3.1. Determination of annealing temperature, primer concentration and IAC copy number

The annealing temperature and concentrations of individual primer pairs were optimized to allow for efficient amplification of the target genes all together. The annealing temperature for the multiplex PCR assay was established at 66°C, while optimum primer concentrations giving the best resolution were found to be 0,4 µM for *gyrB* (F/R), *gapA* (F/R) and *rtxA* (F/R) primers and 0,8 µM for the pSFn1-6020/pSFn1-6974 primer set (data not shown). Using these optimized PCR conditions, a concentration of 284 IAC copies was determined as the lowest number enabling reproducible IAC amplification visible by gel electrophoresis (data not shown).

#### 3.2. Specificity of multiplex PCR assays

In multiplex PCR, all the members of the *V. nigripulchritudo* species ( $n = 45$ ) were successfully detected with the *gyrB* primers, yielding a 257-bp amplicon, irrespective of their isolation place, source and year (Table 1). As indicated in this Table, *V. nigripulchritudo* MP and HP strains ( $n = 28$ ) exhibited the expected band of 257 bp for *gyrB* and 642 bp for *rtxA*, while HP isolates only ( $n = 17$ ) exhibited an additional band of 954 bp corresponding to the plasmid pB1067. All the *V. penaeicida* strains tested ( $n = 18$ ) yielded a 376-bp amplicon for IAC and 504-bp amplicon for *gapA*, regardless of the year or site of isolation. The sequencing of the PCR products confirmed the specificity of the amplification (data not shown). No amplicon, except IAC, was observed in the case of nontarget vibrios ( $n = 10$ ) or other bacterial species ( $n = 4$ ), demonstrating the specificity of the assay.

#### 3.3. Evaluation of the detection limits

The multiplex PCR assay worked efficiently with purified genomic DNA extracted from *V. penaeicida* AM101 and *V. nigripulchritudo* SFn1 individually, as well as in combination. The minimum DNA quantity necessary to detect the 4 PCR amplicons with the same band intensity was determined at 1 pg when IAC DNA was not added to the PCR mix (data not shown), and at 10 pg when using 284 copies of IAC (Figure 1, lane 4). At this concentration, the IAC coamplified with target DNA, as shown in Figure 1. Using 10-fold serial dilutions in artificial seawater or in hemolymph, the detection limits were found to be about 200 cfu/ml for *V. penaeicida* AM101 and *V. nigripulchritudo* SFn1, as determined by the plate count method (Figure 2 A and 2 B, lanes 5). No difference in band intensity was noticed when the multiplex PCR was carried out with DNA extracted from bacterial cell suspensions prepared in seawater or in shrimp hemolymph (data not shown).

### 3.4. Detection of *V. nigripulchritudo* and *V. penaeicida* in experimentally infected shrimp

The applicability of the multiplex PCR to detect these two species in non-symptomatic animals was evaluated using shrimp experimentally infected with these two pathogens and sampled before the onset of mortalities (i.e. 24 hrs post-challenge). In uninfected (control) shrimp, no presumptive *V. nigripulchritudo* isolates (determined as black colonies among total heterotrophic bacteria on MA supplemented with 2% glycerol) could be detected by viable plate counts in hemolymph samples. Presumptive isolates of *V. penaeicida* were not detected, since this method did not permit the phenotypical distinction of this bacterial species among the endogenous microflora. When analyzed by multiplex PCR, these 6 samples were found negative for *V. penaeicida* detection (Supplementary data, Figure S1 A; lanes 1-6). However, one sample (Supplementary data, Figure S1 A; lane 3) exhibited a 257-bp amplicon, indicating the presence of bacterial cells belonging to the *V. nigripulchritudo* species within the microflora of this animal. In shrimp challenged with strain SFn1, presumptive *V. nigripulchritudo* cells ranged between 100 and  $4.10^3$  cfu/ml as determined by the viable plate count method. Multiplex PCR analysis, used to ascertain the presence of this species in infected animals, showed that 4 out of 6 samples (Supplementary data, Figure S1 B; lanes 8, 9, 10 and 12) generated the 3 amplicons corresponding to the expected PCR profile for *V. nigripulchritudo* HP isolates. Considering shrimp challenged with strain AM101, multiplex PCR analysis allowed the amplification of a 504-bp amplicon in 5 out of 6 hemolymph samples (Supplementary data, Figure S1 C; lanes 13, 15, 16, 17 and 18), indicating the presence of *V. penaeicida* cells within the circulatory system of these shrimp. Interestingly, a 257-bp amplicon corresponding to the PCR product obtained in the presence of the *V. nigripulchritudo* species was also detected in 3 of these samples. The IAC was amplified in all samples analyzed by multiplex PCR.

### 3.5. Application of the method to field samples

From January 2008 to December 2010, a total of 94 isolates was collected from moribund shrimp sampled in 10 different farms facing mortality outbreaks (Figure 3). Of these 94 samples, 48.9% were assigned to the *V. nigripulchritudo* species by means of phenotypic tests, while 36.2 % were presumptively identified as belonging to *V. penaeicida* (Table 3). Fourteen strains (14.9%) could not be assigned to any bacterial species because of the lack of resolution power of these tests. A comparison of the multiplex PCR results and phenotypic characterization showed that all the isolates biochemically identified as *V. nigripulchritudo* were also detected as belonging to this bacterial species in PCR, while 4 isolates that could not be determined after phenotypic characterization were identified by multiplex PCR as *V. nigripulchritudo* (Table 3). Of the 34 isolates presumptively identified as *V. penaeicida*, 32 were assigned to this bacterial species in multiplex PCR. On the basis of 16S rRNA gene sequence identity, the samples showing discordant results between the two methods were shown to belong to the *Harveyi* clade of the genus *Vibrio* (data not shown). Among the 50 isolates identified by multiplex PCR as belonging to the *V. nigripulchritudo* species, 26% were characterized as MP, while 32% were detected as HP. MP strains were recovered from 5 farms (i.e. farms A, D, E, I and J), while HP strains were detected in 3 locations (i.e. farms G, I and H). Previous studies showed that supernatant toxicity was a characteristic of HP but not MP strains [18], therefore shrimp were injected with supernatants prepared from these isolates to strengthen our pathotype determination. As shown in Figure 4, 0% mortality was observed one day post-injection in control shrimp, whereas 75-100% cumulative mortality occurred in animals challenged with supernatants prepared from all the strains characterized as HP.

## 4. Discussion

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*V. nigripulchritudo* and *V. penaeicida* tracking is of interest for disease control programs or epidemiological studies concerned with the ecology and natural history of these diseases. However, conventional phenotypic approaches for diagnosis of vibriosis present a number of limitations, such as the time required to obtain confirmatory results, generally too long for practical management decisions, or the great variability in the biochemical characteristics of the *Vibrionaceae*, that renders their phenotypic identification on this basis particularly difficult. Commonly used molecular techniques may provide an efficient means of identifying a bacterial species [26]. However, these typing approaches do not have the ability to discriminate the range of virulence potentials of the *V. nigripulchritudo* species. To simultaneously detect these bacterial species and allow for pathotype discrimination, we therefore combined in a multiplex PCR assay two species-specific primer sets along with *V. nigripulchritudo* virulence biomarkers. Critical parameters of such an assay rely on the choice of the target genes and the design of corresponding primers. Because of lateral gene transfers, hidden paralogy or chromosomal rearrangements, any phylogenetic marker for the identification of *Vibrio spp* will have weaknesses and strengths [26]. For instance, the 16S rRNA gene has been widely used for species definition and identification among bacteria, but is of limited value in *Vibrio spp* because of its low taxonomic resolution [11]. As alternative taxonomic markers, we therefore selected *gyrB* and *gapA*, two highly conserved housekeeping genes commonly found in vibrios and previously demonstrated to possess the discriminating potential for the development of species-specific PCR primers [17, 9]. The results obtained with these 2 primer sets were shown to be accurate and highly specific to their corresponding target species, since no band was amplified from any of the nontarget *Vibrio spp* or other tested species. To screen the virulence potentials of *V. nigripulchritudo* strains, we also included in the assay primers targeting two different plasmids. The first primer set was previously designed and specifically amplifies a 954-bp fragment of plasmid pB1067, only found in HP strains [27], while the second set targets a *rtxA* toxin gene, located on a larger replicon, pA1066, and found in HP and MP strains only [18]. We used a collection of 45 *V. nigripulchritudo* strains, whose virulence patterns were previously determined, and 18 *V. penaeicida* isolates to validate the pathotyping ability and specificity of our multiplex PCR. Data presented herein demonstrate that this method shows a very efficient discriminating potential, since our results matched exactly with those obtained in a previous study by means of experimental challenges [6]. Among the limitations to using PCR as a diagnostic tool, there is the risk of DNA contamination, that can induce false-positive reactions, and also the presence of inhibitory substances found in biological samples or in the environment that may produce false-negative PCR result [28]. To avoid this potential bias, we synthesized and integrated in the assay an IAC competitive with the *gyrB* gene of *V. nigripulchritudo* generating a control signal even when there is no target sequence present. The drawback of this strategy is that the IAC may decrease the amplification efficiency and thus the detection limit of the reaction [1, 13]. In our assay, we established that 284 copies was the lowest concentration enabling reproducible IAC amplification visible by gel electrophoresis. At this concentration, a slight competition between IAC and the target DNA occurred, as we observed a 10-fold lowering of the detection limit. However, even with the presence of the IAC, we showed that the sensitivity of our PCR (established at 10 pg purified DNA and 200 cfu/ml) was consistent with the multiplex PCR methods described for the identification of other vibrios (between 1 and 10<sup>3</sup> cfu/ml or between 10 and 100 pg for DNA) [4, 20, 12, 25].

We tested the effectiveness of our method using hemolymph samples from experimentally challenged prawns. We showed that the multiplex PCR evidenced *V. penaeicida* as well as *V. nigripulchritudo* HP cells in infected animals before the onset of mortality. The presence of isolates belonging to *V. nigripulchritudo* was also detected in uninfected control shrimp as well as in shrimp challenged with *V. penaeicida*, while no putative *V. nigripulchritudo* colonies could be enumerated on the corresponding glycerol-supplemented marine agar plates. Since

it as been previously reported that 30% of the bacterial flora of apparently healthy shrimp was *Vibrio* sp. [3], it is not surprising to detect isolates from the *V. nigripulchritudo* species in the shrimp circulatory system. Such results exemplify the need of pathotyping, since the detection of *V. nigripulchritudo* isolates is not necessarily linked to pathogenicity, as previously reported [6].

Finally we used the multiplex PCR to evaluate the occurrence of these two bacterial species using field samples collected from shrimp farms encountering mortality peaks from January 2008 to December 2010. We showed that 87.2% of the isolates could be identified by our assay as *V. penaeicida* or *V. nigripulchritudo*, confirming the major epidemiological importance of these 2 species in recurring mortality events of cultured shrimp in New-Caledonia. Supporting previous works [10, 19], we also observed a clear seasonal isolation pattern. The isolation rate of *V. nigripulchritudo* strains (72%) was highly correlated with the summer period, and thus the water temperature, while 80% of the isolates collected during the southern winter were identified as *V. penaeicida*. Such strong relationship has been documented for other *Vibrio* species [21, 14] and temperature changes are considered as one of the most frequently used cues controlling the expression of numerous virulence factors in pathogenic bacteria [16]. However, it remains to be investigated whether this environmental signal plays some role in vibrio abundance or may modulate virulence factor expression in *V. penaeicida* and *V. nigripulchritudo*. As of 2007, the virulent lineage of *V. nigripulchritudo* HP strains implicated in the 'Summer Syndrome' was evidenced in one particular area of New Caledonia (i.e. the Saint Vincent Bay, an estuarine area of the southwestern lagoon), causing the disease in 3 farms only, including farms G and H. The multiplex PCR allowed us to confirm the presence of the HP pathotype in these two locations but also to report, for the first time, its occurrence in another neighbouring farm (farm I), leading us to suspect the disease extension. Bacterial supernatant toxicity being a HP-specific feature [18], we confirmed our pathotype determination by *in vivo* experimental infection and showed congruent results between the two approaches. The results of this 3-year field study also revealed the yearly recurrence of HP isolates in the environment of these facilities. To date, the environmental reservoir of *V. nigripulchritudo* remains to be determined, although shrimp pond sediments are suspected to represent an ecosystem favoring bacterial persistence between crops in the affected farms and/or pathogen transmission to the host [6, 27]. Finally, our multiplex PCR assay identified MP strains in both 'Summer Syndrome'-affected and non-affected farms all along the West coast of the island, confirming previous data indicating that pathogenic and non-pathogenic strains co-exist in the same environment and at the same time [9].

To conclude, this assay represents a simple, rapid and cost-effective tool for diagnosis and implementing timely risk management decisions. This should not only help epidemiologists to better estimate the incidence of *Vibrio* infections in aquaculture systems and the associated risk of spreading of these diseases, but also provide an efficient tool for ecologists to understand the seasonal and geographical distribution as well as the effects of environmental parameters (such as temperature) on these two pathogens and associated diseases.

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## Tables

Table 1: Specificity of detection using primers for *gyrB*, *gapA*, *rtxA* and a 954-bp fragment of plasmid pB1067 tested on clinical and environmental isolates of *V. nigripulchritudo* and *V. penaeicida* as well as others *Vibrio* spp. and non-*Vibrio* isolates using optimized multiplex PCR. Note: [a] ATCC, American Type Culture Collection; CIP, Collection de l'Institut Pasteur; LMG, Laboratorium voor Microbiologie, Gent [b] virulence for *L. stylirostris*: NP, non-pathogenic (0-20% mortality); MP, moderately pathogenic (20–80%); HP, highly pathogenic (80–100%) [c] (+) indicates a positive amplification, (-) a negative amplification, IAC: Internal Amplification Control.

species	source	isolate name <sup>a</sup> and pathotype <sup>b</sup>	Multiplex PCR results <sup>c</sup>				
			IAC	<i>pB1067</i>	<i>rtxA</i>	<i>gyrB</i>	<i>gapA</i>
<i>Vibrio nigripulchritudo</i>	Reference strain	CIP 103336 <sup>T</sup>	-	-	-	+	-
	field isolates sampled in New Caledonian shrimp farms	<b>highly pathogenic (HP)</b> : SFn1, SFn2, SFn27, SFn49, SFn105, SFn106, SFn127, SFn128, SFn135, POn2, POn19, AgMn7, AgMn8, AgMn9, AgMn10, AgMn12, AgMn13	-	+	+	+	-
		<b>moderately pathogenic (MP)</b> : AQn1, AQn2, BDn1, BDn2, BLFn1, BLFn2, ENn2, Fn1, MT1, Wn1, Wn13	-	-	+	+	-
		<b>non pathogenic (NP)</b> : AgMn1, AgMn3, ENn1, ESn2, Fn2, FTn1, POn4, POn10, POn12, POn13, SFn115, SFn118, SOn1, SOn2, SFn115, SFn118	+	-	-	+	-
<i>Vibrio penaeicida</i>	Reference strain	KH-1 <sup>T</sup>	+	-	-	-	+
	field isolates sampled in New Caledonian shrimp farms	AM101, AM107, AM108, AQ103, AQ105, AQ112, AQ120, BD1, BD2, BD3, BLF2, F5, PO1, PO2, SB2, SF140, SV4	+	-	-	-	+
<i>Vibrio splendidus</i>	Reference strain	LMG4042T	+	-	-	-	-
<i>Vibrio tasmaniensis</i>	Reference strain	LMG20012T	+	-	-	-	-
<i>Vibrio pelagius</i>	Reference strain	LMG3897T	+	-	-	-	-
<i>Vibrio chagasii</i>	Reference strain	LMG16745	+	-	-	-	-
<i>Vibrio pacinii</i>	Reference strain	LMG19999	+	-	-	-	-
<i>Vibrio harveyi</i>	Reference strain	LMG7890	+	-	-	-	-
<i>Vibrio carchariae</i>	Reference strain	02/001	+	-	-	-	-
<i>Vibrio nereis</i>	Reference strain	LMG3895T	+	-	-	-	-
<i>Vibrio cyclitrophicus</i>	Reference strain	LMG21359T	+	-	-	-	-
<i>Vibrio orientalis</i>	Reference strain	LMG7897	+	-	-	-	-
<i>Escherichia coli</i>	Reference strain	ATCC 25922	+	-	-	-	-
<i>Pseudomonas aeruginosa</i>	Reference strain	ATCC 27853	+	-	-	-	-
<i>Staphylococcus aureus</i>	Reference strain	ATCC 25923	+	-	-	-	-
<i>Enterococcus faecalis</i>	Reference strain	ATCC 29212	+	-	-	-	-

Table 2: IAC and primers used for multiplexed PCR detection and typing of *V. nigripulchritudo* and *V. penaeicida*. Sequences of the IAC primer set (IACf and IACr) which are identical to the *rtxA* gene primer set (*rtxA* F and *rtxA* R) are identified in bold, while the sequence designed to amplify a 376-bp region of pUC19 is italicized.

Primer	Sequence (5'– 3')	Target species and gene	product size (bp)	References
gapA F gapA R	ACTGCACGTAAGCACATCACTGCG TAGAACGCCCTTTAGAGGACCT	<i>V. penaeicida</i> glyceraldehyde-3-phosphate dehydrogenase gene ( <i>gapA</i> )	504	This study
gyrB F gyrB R	AAAACCCGAACGAAGCGAAAAC ACCTTTCAGTGGCAAGATGGCT	<i>V. nigripulchritudo</i> DNA gyrase gene ( <i>gyrB</i> )	257	This study and [9]
rtxA F rtxA R	TTGGCGGGTAAGCACCCCGA GTCGGAGACACCGGTGCGTT	<i>V. nigripulchritudo</i> pathotype MP and HP RTX A gene ( <i>RtxA</i> )	642	This study
pSFn1-6020 pSFn1-6974	TGTCTTCTGGATCGCTTCGCC CGTCGTAAGGAGCGATAAGCC	<i>V. nigripulchritudo</i> pathotype HP 954 bp fragment from plasmid pB1067	954	[27]
IACf IACr	<b>TTGGCGGGTAAGCACCCCGAGCTCTCCGCTTCCTCGCTCA</b> <b>GTCGGAGACACCGGTGCGTTATCCGGTAAGCGGCAGGGTC</b>	Internal amplification control (IAC)	376	This study

Table 3: Detection of *V. penaeicida* and *V. nigripulchritudo* by culture-based methods and multiplex PCR in moribund shrimp sampled during mortality outbreaks occurring in 10 different shrimp farms of New Caledonia between January 2008 and December 2010. Note: ND, not determined; MP, moderately pathogenic; HP, highly pathogenic

Farm name	sampling date	Isolate name	Presumptive phenotypic identification	Multiplex PCR identification
Farm A	08/04/2008	IFNC08020	<i>V. nigripulchritudo</i>	<i>V. nigripulchritudo</i> <b>MP</b>
	08/04/2008	IFNC08021	<i>V. nigripulchritudo</i>	<i>V. nigripulchritudo</i> <b>MP</b>
	22/10/2009	IFNC09030	ND	<b>negative</b>
		IFNC09031	ND	<b>negative</b>
		IFNC09032	ND	<b>negative</b>
		IFNC09035	ND	<b>negative</b>
26/10/2009	IFNC09036	ND	<b>negative</b>	
	IFNC09038	ND	<b>negative</b>	
Farm B	08/12/2010	IFNC10045	<i>V. penaeicida</i>	<i>V. penaeicida</i>
		IFNC09039	ND	<b>negative</b>
Farm C	23/01/2008	IFNC08001	<i>V. nigripulchritudo</i>	<i>V. nigripulchritudo</i>
		IFNC08002	<i>V. nigripulchritudo</i>	<i>V. nigripulchritudo</i>
		IFNC08003	ND	<i>V. nigripulchritudo</i>
		IFNC08004	<i>V. nigripulchritudo</i>	<i>V. nigripulchritudo</i>
	21/01/2009	IFNC09005	<i>V. nigripulchritudo</i>	<i>V. nigripulchritudo</i>
		IFNC09006	<i>V. nigripulchritudo</i>	<i>V. nigripulchritudo</i>
	23/03/2010	IFNC10027	<i>V. nigripulchritudo</i>	<i>V. nigripulchritudo</i>
		IFNC10025	<i>V. penaeicida</i>	<i>V. penaeicida</i>
	24/03/2010	IFNC10026	<i>V. nigripulchritudo</i>	<i>V. nigripulchritudo</i>
		IFNC10024	<i>V. penaeicida</i>	<i>V. penaeicida</i>
27/04/2010	IFNC10028	<i>V. penaeicida</i>	<i>V. penaeicida</i>	
	IFNC10032	<i>V. penaeicida</i>	<i>V. penaeicida</i>	
Farm D	10/03/2010	IFNC10033	<i>V. penaeicida</i>	<i>V. penaeicida</i>
		IFNC10029	<i>V. nigripulchritudo</i>	<i>V. nigripulchritudo</i> <b>MP</b>
Farm E	28/10/2008	IFNC08037	<i>V. penaeicida</i>	<i>V. penaeicida</i>
		IFNC08038	<i>V. penaeicida</i>	<i>V. penaeicida</i>
	04/06/2009	IFNC09018	<i>V. penaeicida</i>	<i>V. penaeicida</i>
		IFNC09019	<i>V. penaeicida</i>	<i>V. penaeicida</i>
	11/06/2009	IFNC09020	<i>V. penaeicida</i>	<i>V. penaeicida</i>
		IFNC09021	<i>V. penaeicida</i>	<i>V. penaeicida</i>
	01/10/2009	IFNC09024	<i>V. penaeicida</i>	<b>negative</b>
		IFNC09025	<i>V. penaeicida</i>	<i>V. penaeicida</i>
	16/10/2009	IFNC09042	<i>V. penaeicida</i>	<i>V. penaeicida</i>
		IFNC09043	<i>V. penaeicida</i>	<i>V. penaeicida</i>
20/03/2010	IFNC10018	<i>V. penaeicida</i>	<i>V. penaeicida</i>	
	IFNC10019	<i>V. penaeicida</i>	<i>V. penaeicida</i>	
	IFNC10020	<i>V. penaeicida</i>	<i>V. penaeicida</i>	
21/03/2010	IFNC10021	<i>penaeicida</i>	<i>penaeicida</i>	
	IFNC10022	<i>V. nigripulchritudo</i>	<i>V. nigripulchritudo</i> <b>MP</b>	
	IFNC10023	<i>V. nigripulchritudo</i>	<i>V. nigripulchritudo</i> <b>MP</b>	
08/05/2010	IFNC10037	<i>V. penaeicida</i>	<i>V. penaeicida</i>	
	IFNC10038	<i>V. penaeicida</i>	<i>V. penaeicida</i>	
	IFNC10039	<i>V. penaeicida</i>	<i>V. penaeicida</i>	
Farm F	22/10/2009	IFNC10044	<i>V. penaeicida</i>	<b>negative</b>
		IFNC09033	ND	<b>negative</b>
IFNC09034		ND	<b>negative</b>	
Farm G	14/01/2009	IFNC09037	ND	<b>negative</b>
		IFNC09001	<i>V. nigripulchritudo</i>	<i>V. nigripulchritudo</i> <b>HP</b>

		IFNC09002 IFNC09003 IFNC09004	<i>V. nigripulchritudo</i> <i>V. nigripulchritudo</i> <i>V. nigripulchritudo</i>	<i>V. nigripulchritudo</i> <b>HP</b> <i>V. nigripulchritudo</i> <b>HP</b> <i>V. nigripulchritudo</i> <b>HP</b>
<b>Farm H</b>	26/04/2010	IFNC10030	<i>V. nigripulchritudo</i>	<i>V. nigripulchritudo</i> <b>HP</b>
		IFNC10031	<i>V. nigripulchritudo</i>	<i>V. nigripulchritudo</i> <b>HP</b>
	24/03/2009	IFNC09014	<i>V. nigripulchritudo</i>	<i>V. nigripulchritudo</i> <b>HP</b>
		IFNC09015	<i>V. nigripulchritudo</i>	<i>V. nigripulchritudo</i> <b>HP</b>
<b>Farm I</b>	25/02/2008	IFNC08005	<i>V. nigripulchritudo</i>	<i>V. nigripulchritudo</i>
		IFNC08006	<i>V. nigripulchritudo</i>	<i>V. nigripulchritudo</i>
		IFNC08007	<i>V. nigripulchritudo</i>	<i>V. nigripulchritudo</i>
		IFNC08008	<i>V. nigripulchritudo</i>	<i>V. nigripulchritudo</i>
		IFNC08009	ND	<i>V. nigripulchritudo</i>
		IFNC08010	ND	<i>V. nigripulchritudo</i>
	20/03/2008	IFNC08014	<i>V. nigripulchritudo</i>	<i>V. nigripulchritudo</i>
		IFNC08015	<i>V. nigripulchritudo</i>	<i>V. nigripulchritudo</i>
		IFNC08016	<i>V. nigripulchritudo</i>	<i>V. nigripulchritudo</i>
		IFNC08017	<i>V. nigripulchritudo</i>	<i>V. nigripulchritudo</i>
		IFNC08018	<i>V. nigripulchritudo</i>	<i>V. nigripulchritudo</i>
	08/04/2008	IFNC08024	<i>V. nigripulchritudo</i>	<i>V. nigripulchritudo</i> <b>MP</b>
		IFNC08025	<i>V. nigripulchritudo</i>	<i>V. nigripulchritudo</i> <b>MP</b>
	26/05/2008	IFNC08026	<i>V. nigripulchritudo</i>	<i>V. nigripulchritudo</i> <b>HP</b>
IFNC08027		<i>V. nigripulchritudo</i>	<i>V. nigripulchritudo</i> <b>MP</b>	
IFNC08028		<i>V. penaeicida</i>	<i>V. penaeicida</i>	
17/10/2008	IFNC08035	<i>V. penaeicida</i>	<i>V. penaeicida</i>	
	IFNC08036	<i>V. penaeicida</i>	<i>V. penaeicida</i>	
30/12/2008	IFNC08044	<i>V. nigripulchritudo</i>	<i>V. nigripulchritudo</i> <b>MP</b>	
	IFNC08045	<i>V. nigripulchritudo</i>	<i>V. nigripulchritudo</i> <b>MP</b>	
28/01/2009	IFNC09007	<i>V. nigripulchritudo</i>	<i>V. nigripulchritudo</i> <b>MP</b>	
	IFNC09008	<i>V. nigripulchritudo</i>	<i>V. nigripulchritudo</i> <b>MP</b>	
23/12/2009	IFNC10001	<i>V. penaeicida</i>	<i>V. penaeicida</i>	
	IFNC10002	<i>V. penaeicida</i>	<i>V. penaeicida</i>	
	IFNC10003	<i>V. penaeicida</i>	<i>V. penaeicida</i>	
	IFNC10004	<i>V. penaeicida</i>	<i>V. penaeicida</i>	
03/03/2010	IFNC10008	<i>V. nigripulchritudo</i>	<i>V. nigripulchritudo</i> <b>HP</b>	
	IFNC10009	<i>V. nigripulchritudo</i>	<i>V. nigripulchritudo</i> <b>HP</b>	
	IFNC10010	<i>V. nigripulchritudo</i>	<i>V. nigripulchritudo</i> <b>HP</b>	
	IFNC10011	<i>V. nigripulchritudo</i>	<i>V. nigripulchritudo</i> <b>HP</b>	
09/03/2010	IFNC10012	<i>V. nigripulchritudo</i>	<i>V. nigripulchritudo</i> <b>HP</b>	
	IFNC10013	<i>V. nigripulchritudo</i>	<i>V. nigripulchritudo</i> <b>HP</b>	
	IFNC10014	<i>V. nigripulchritudo</i>	<i>V. nigripulchritudo</i> <b>HP</b>	
	IFNC10015	<i>V. penaeicida</i>	<i>V. penaeicida</i>	
	IFNC10016	<i>V. penaeicida</i>	<i>V. penaeicida</i>	
	IFNC10017	<i>V. penaeicida</i>	<i>V. penaeicida</i>	
<b>Farm J</b>	09/04/2008	IFNC08022 IFNC08023	ND <i>V. nigripulchritudo</i>	<i>V. nigripulchritudo</i> <i>V. nigripulchritudo</i> <b>MP</b>

## Figures

Figure 1: detection limits of the multiplex PCR. DNA extracted from pure cultures of *V. nigripulchritudo* SFn1 and *V. penaeicida* AM101 was coamplified with IAC using multiplex PCR. Genomic DNA was serially diluted tenfold from 10 ng to 0.1 pg and amplified by PCR with a defined IAC concentration (284 copies in every reaction). The amplicons corresponding to *gyrB*, *gapA*, *rtxA* and to a fragment of plasmid pB1067, with predicted sizes of 257, 504, 642 and 954 bp respectively, are indicated by yellow arrows. Lane M, 100 bp plus DNA ladder (GelPilot, QIAGEN); lanes 1-6, varying concentrations of a combination of *V. nigripulchritudo* SFn1 and *V. penaeicida* AM101 DNA (lane 1, 10 ng; lane 2, 1 ng; lane 3, 0.1 ng; lane 4, 10 pg, lane 5, 1 pg; lane 6, 0.1 pg); lane 7, no DNA template (negative control); lane 8, IAC DNA template (positive control).

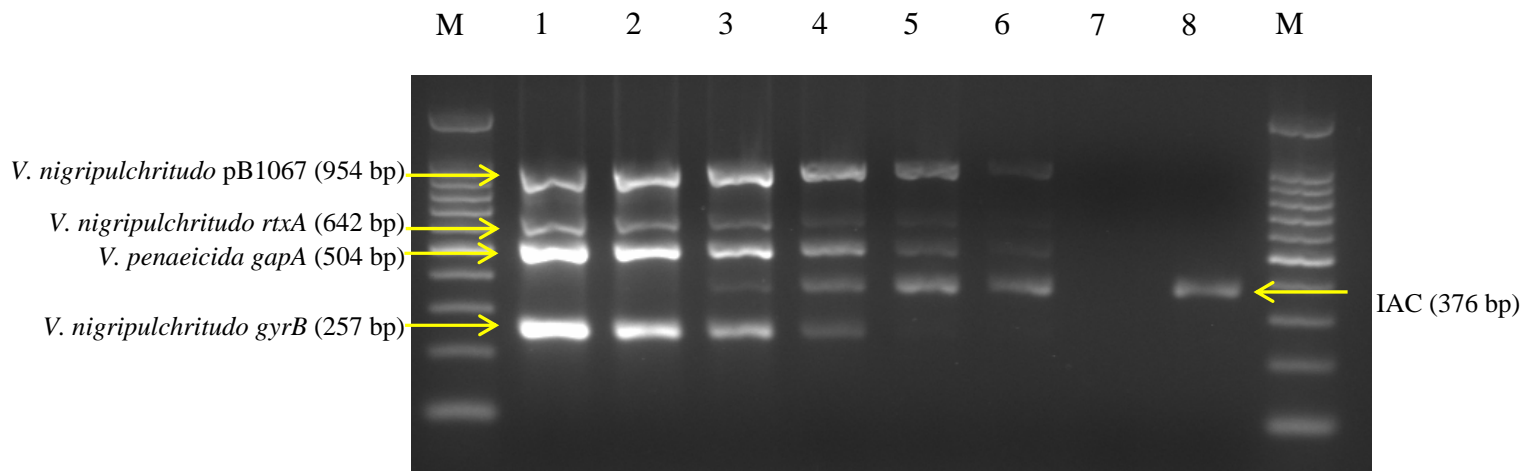


Figure 2: DNA extracted from *V. penaeicida* AM101 (A) or *V. nigripulchritudo* SFn1 (B) bacterial cells suspended in hemolymph of naïve shrimp and coamplified with IAC using multiplex PCR. Bacterial cells were serially diluted tenfold in hemolymph and the extracted DNA was amplified by PCR with a defined IAC concentration (284 copies). Lane M, 100 bp plus DNA ladder (GelPilot, QIAGEN); lanes 1-7, PCR products amplified from DNA extracted from 10-fold dilutions (from  $2 \cdot 10^6$  to 2 cfu/ml); lane 8, no DNA template (negative control); lane 9, IAC DNA template (positive control).

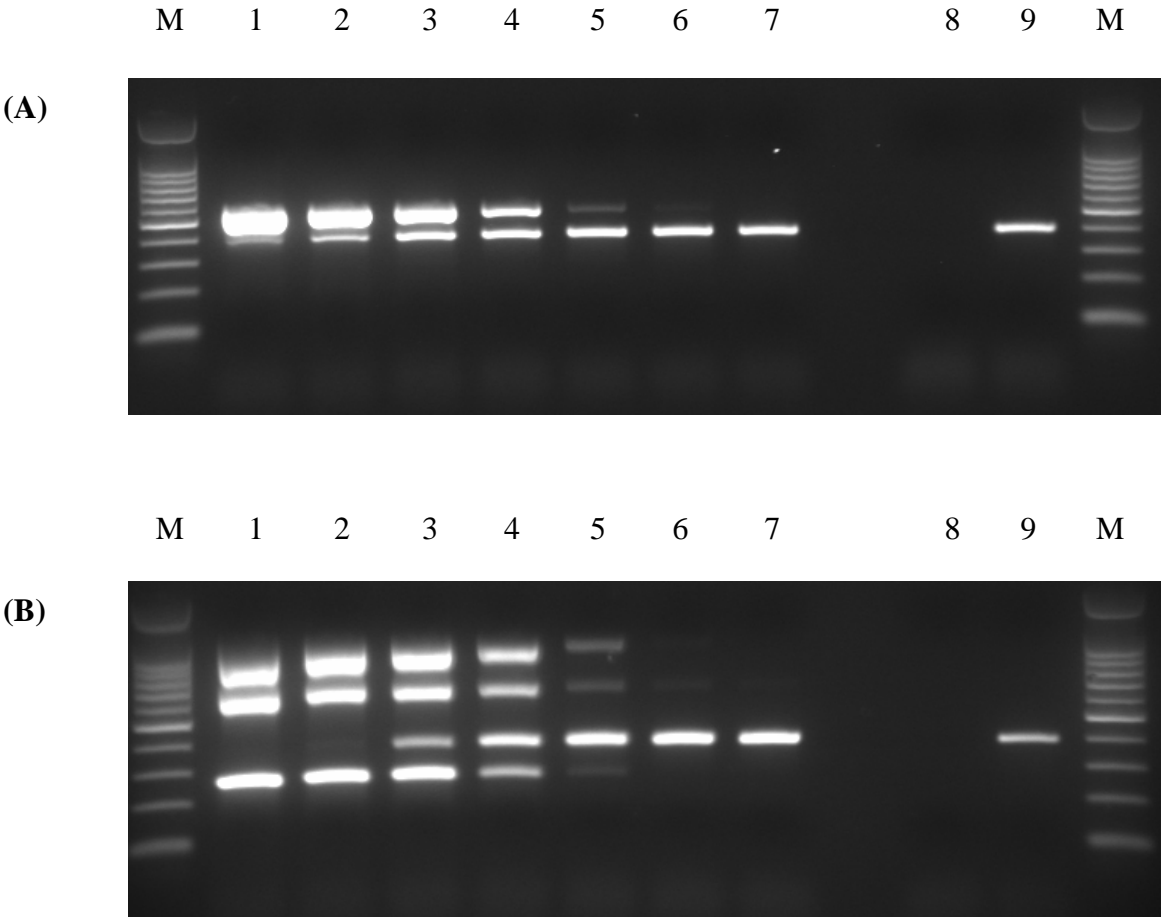




Figure 3: Map of New-Caledonia indicating the geographical localization of the 10 shrimp farms (designated A to J) sampled in this study.

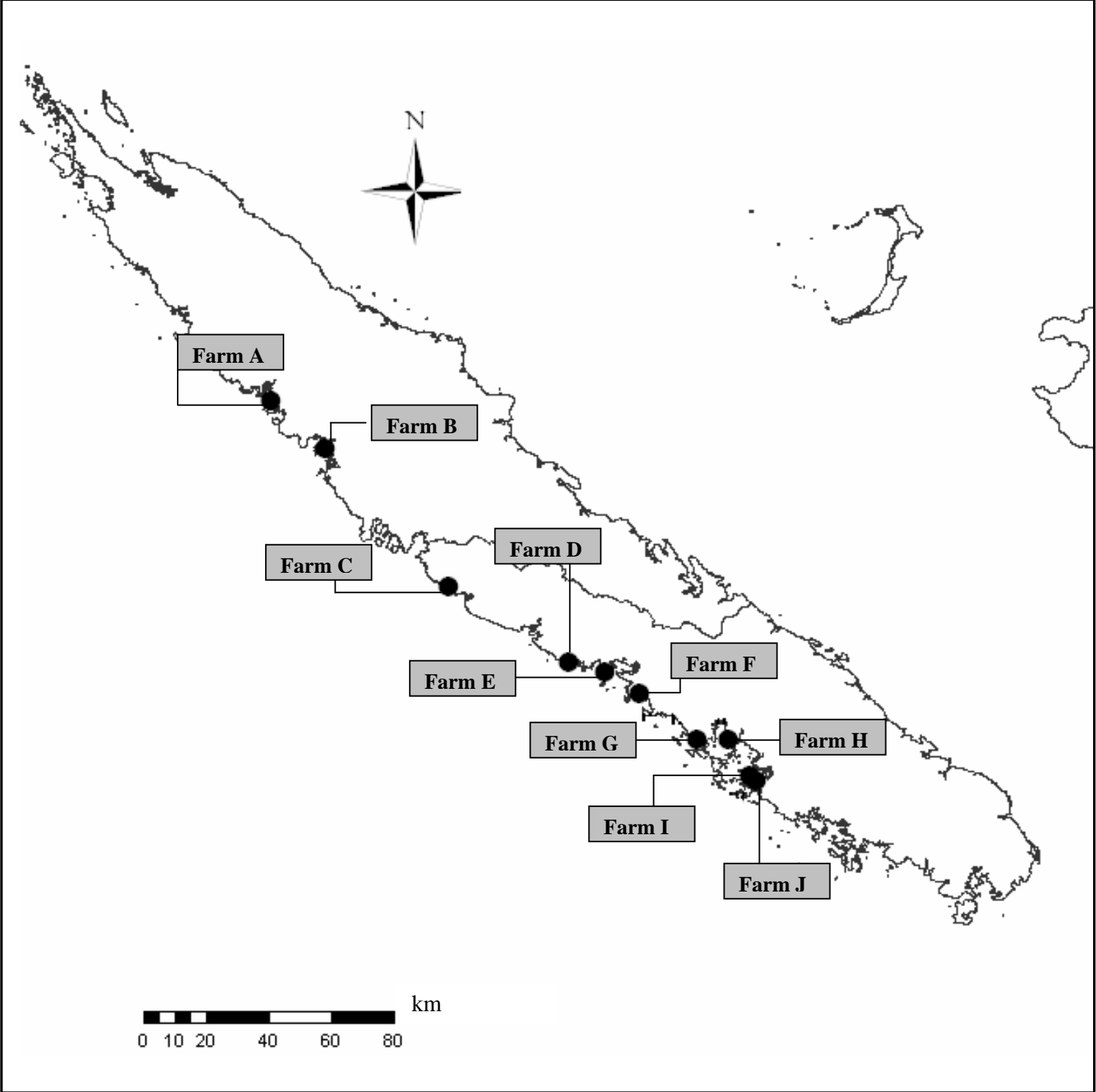
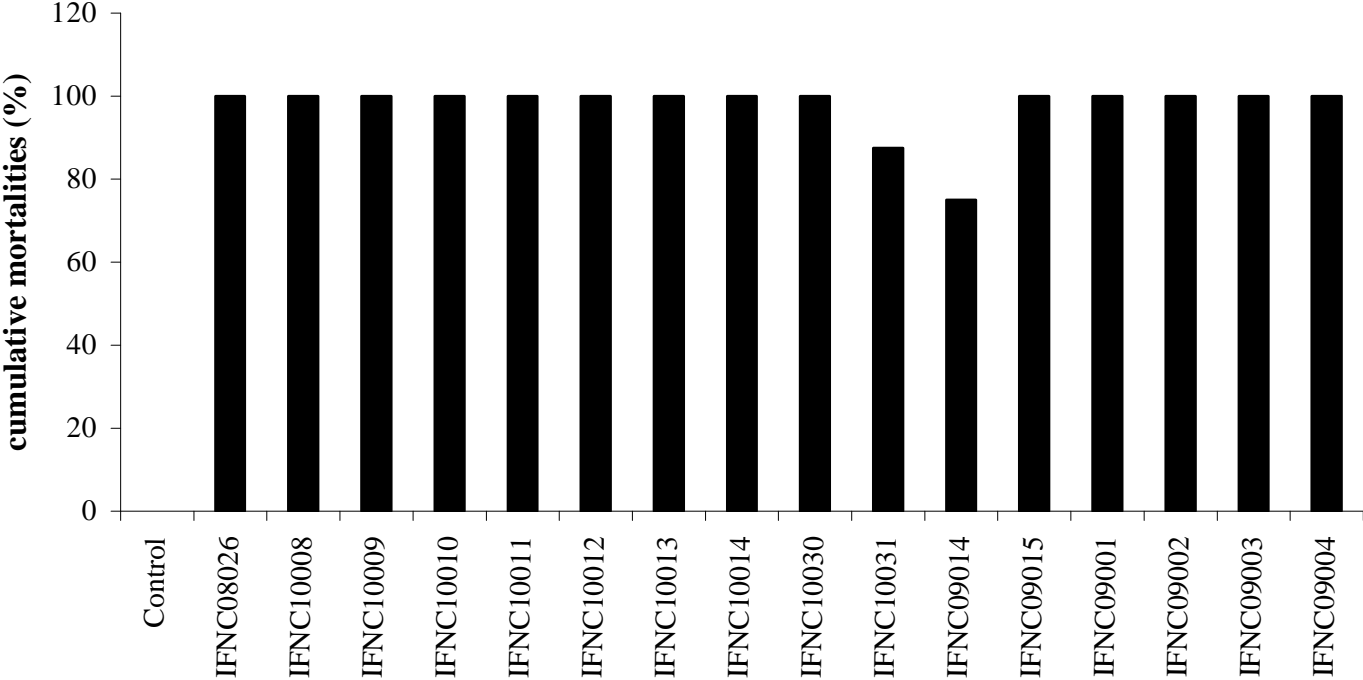


Figure 4: Shrimp mortality in response to injection of bacterial culture supernatants. Animals (n = 8) were injected with 100 µl of bacterial supernatant prepared from overnight cultures grown in MB 5X as described previously [18]. Survival was assessed after 24 h.



## Supplementary data

Figure S1: multiplex PCR-based detection of *V. nigripulchritudo* strain SFn1 and *V. penaeicida* strain AM101 from the hemolymph of experimentally infected shrimp. Lane M, 100 bp plus DNA ladder (GelPilot, QIAGEN); lanes 1-6, PCR products of purified DNA extracted from control (unchallenged) shrimp; lanes 7-12, PCR products of purified DNA extracted from shrimp infected by *V. nigripulchritudo* SFn1; lanes 13-18, PCR products of purified DNA extracted from shrimp infected by *V. penaeicida* AM101; (-) no template control; (+) IAC in the absence of bacterial genomic DNA.

