Amplified fragments of ornithine decarboxylase gene, *toxR*, and an unknown gene marker could distinguish strains of *Vibrio harveyi* and *Vibrio campbellii* implicated in shrimp disease

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Three strains of Vibrios have been reported to cause shrimp disease, type strain of *Vibrio harveyi* and *Vibrio campbellii*, and a variant strain of *Vibrio harveyi* that was described to exhibit significant sequence difference of *toxR* and *hemolysin* genes from type strain *V. harveyi*. The early detection of these Vibrios causing shrimp disease is necessary to prevent disease outbreak. This study was focused on the use of three sets of PCR primers that target genes for ornithine decarboxylase, ToxR, and an unknown gene to detect and distinguish type strains of *V. harveyi* and *V. campbellii* and the variant *V. harveyi* that are pathogenic to black tiger shrimp (*Penaeus monodon*). Multiplex PCR profile revealed two bands (a 900-bp fragment of ornithine decarboxylase gene and a 175-bp fragment of an unknown gene) for type strain *Vibrio harveyi* (NBRC 15634), one band representing a 245-bp *toxR* gene fragment for type strain *Vibrio campbellii* (NBRC 15631), and two bands (a 900-bp *odc* gene fragment and a 245-bp *toxR* gene fragment) from isolate SW-9702, a representative of the variant *V. harveyi*. By using digoxigenin-labeled dUTPs in multiplex PCR, amplicons are labeled and could serve as probes for hybridization with blots containing known gene templates to confirm the amplification of target genes. The 900-bp labeled amplicons from type strain and variant *V. harveyi* hybridized with the *V. harveyi odc* gene blot. As expected, the labeled 175-bp amplicon of type strain *V. harveyi* hybridized with the blotted 175-bp putative marker of type strain *V. harveyi*, while the *toxR* amplicon from *V. campbellii* hybridized with blotted *toxR* of type strain *V. campbellii*. Hybridization signal of the *V. campbellii toxR* blot with the multiplex PCR products from type strain *V. harveyi* was much reduced compared to the signal observed with multiplex PCR products from the variant strain of *V. harveyi*, because *V. campbellii toxR* exhibits only 76% sequence similarity with *toxR* of type strain *V. harveyi* and 92-93% similarity with the variant strain of *V. harveyi*. Results of multiplex PCR with reverse hybridization, as an indirect means to confirm the amplification of target genes, could distinguish the shrimp pathogenic strains of Vibrios used in the study.

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INTRODUCTION

The Philippines, one of the major producers of shrimp in Asia, attained its highest produce in 1994 with 90,000 mt making it the third largest producer in the world (Lavilla-Pitogo 1996). However, the overall production experienced a steady state of decline starting in 1995 due to disease problems, including vibriosis. Vibriosis is a disease that refers to all types of infections caused by bacteria of the genus *Vibrio* (Lavilla-Pitogo

Table 1. Primers used in this study.

Primer	Annealing Temp. (°C)	Target Gene	Size (bp)	Target Sample	Source of Primers
Spef-F1 / Spef-R	59	*odc*	900	Type strain and variant *V. harveyi*	Hedreyda and Rañoa 2007
Vh200-F / Vh200-R	59	Putative marker	175	*V. harveyi*	Conejero and Hedreyda 2003
VcatoxR-F / VcatoxR-R	65	*toxR*	245	*V. campbellii*	Castroverde et al. 2006

and de la Peña 1998). It is necessary to detect the presence of all pathogenic species present in shrimp at an early stage to prevent outbreaks of the disease.

Two *Vibrio* species, *V. harveyi* and *V. campbellii*, are commonly isolated from infected shrimps and have been reported to be pathogenic to shrimp (de la Peña *et al.* 2001). Ten Philippine *Vibrio* isolates that were pathogenic to shrimp are phenotypically similar to *V. harveyi* and *V. campbellii* based on conventional identification tests. These isolates represent a variant strain of *V.harveyi* that possess ornithine decarboxylase and luciferase genes with 96-98% and 96-97% sequence similarity to type strain *V. harveyi*, respectively (Rañoa 2006; Hedreyda and Rañoa 2007). These isolates are considered variants because they were observed to possess *toxR* and hemolysin genes that exhibit higher sequence similarity (92-93% and 92-97%, respectively) with type strain *V. campbellii* than with type strain *V. harveyi* (74-75% and 82-85%, respectively; Rañoa and Hedreyda 2005; Rañoa 2006). A detection protocol should be able to detect the presence of all three strains implicated in shrimp disease.

Previous studies have reported and developed protocols for the detection of these pathogens targeting *toxR* and hemolysin genes (Conejero and Hedredya 2003; Conejero and Hedredya 2004; Rañoa and Hedreyda 2005; Castroverde et al. 2006). The *toxR* gene that codes for a transcription regulatory protein ToxR is implicated in regulating the expression of virulence factors in Vibrios including hemolysin production (Lee et al. 2000; Lin et al. 1993; Osorio and Klose 2000). In this study, detection of *toxR* is also used and the study reports for the first time the detection of the ornithine decarboxylase gene (*odc*) that has been isolated and sequenced from *V. harveyi* but which has not been detected in *V. campbellii* (Hedreyda and Rañoa 2007). The ornithine decarboxylase assay (that is positive for type strain *V. harveyi* and negative for type strain *V. campbellii*) has been used as the only conventional test to differentiate the two species (Bryant et al. 1986; de la Peña et al. 2001). Detection of the *odc* gene could be considered an alternative molecular approach. Another primer set targets the detection of a type strain *V. harveyi*-specific gene fragment (Conejero and Hedreyda 2003) that is not homologous to any known gene.

In this study, amplification of the type strain *V. harveyi* ornithine decarboxylase gene was used to detect the presence of type strain and variant *V. harveyi*. A 175-bp type strain *V. harveyi* -specific gene marker (Conejero and Hedreyda 2003), that could not be amplified in *V. campbellii* nor in the variant strain of *V. harveyi*, was also included to distinguish type strain *V. harveyi* from the other two. The third primer set targets a *V. campbellii*-specific variable region of *toxR* (Castroverde et al. 2006) that was reported in an earlier study. Moreover, the study involves the use of reverse hybridization as an alternative to DNA sequence analysis to confirm that the amplicons are indeed the target genes when detecting the pathogens in several samples.

MATERIALS AND METHODS

Bacterial strains used

Type strains *Vibrio harveyi* (NBRC 15634) and *Vibrio campbellii* (NBRC 15631) used in this study were kindly provided by the National Institute of Technology and Evaluation (NITE) – Biological Resource Center (NBRC), Japan. One of 10 Philippine *Vibrio* isolates designated as strain SW-9702 (de la Peña et al. 2001) is a representative variant that was kindly provided by Dr. de la Peña from Southeast Asian Fisheries Development Center (SEAFDEC), Tigbauan, Iloilo in the Philippines. The *Vibrio* samples were routinely grown in nutrient broth with 1.5% NaCl, incubated overnight at 28°C at 222 rpm, and in nutrient agar media with 1.5% NaCl, incubated at room temperature.

DNA extraction and visualization

Genomic DNA from all Vibrios used was extracted using the commercially available Wizard® Genomic DNA Purification Kit following the manufacturer's protocol (Promega Corporation, USA, 2005).

Selection of primers for detection

Primer pairs that could produce distinguishable amplicon profiles for type strain *V. harveyi* and *V. campbellii* and the variant *V. harveyi* strain were selected from several primer pairs designed in previous studies from the Molecular Microbiology Laboratory in the National Institute of Molecular Biology and Biotechnology, University of the Philippines, Diliman, Quezon

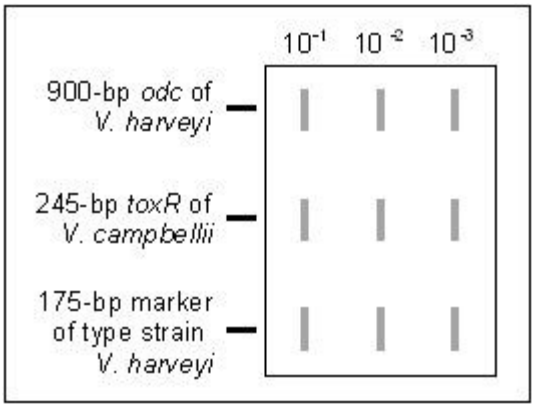

Figure 1. An illustration of how the hybridization membrane was prepared with 10^{-1}, 10^{2}, and 10^{-3} dilutions of known *Vibrio* gene markers.

City, Philippines (Table 1).

Single PCR amplification of the selected gene markers

Preliminary single PCR reactions were performed using each set of primers to verify the conditions for PCR and to confirm the generation of expected size amplicons from three Vibrios used in the study. PCR reactions (20 µl) containing 1.0 µl of the DNA extract from each of three *Vibrio* isolates used, 1x PCR buffer with $MgCl_2$, 0.2 mmol l^{-1} nucleotides, 0.5 mmol l^{-1} of each primer set, and 0.025 U μl^{-1} of GoTaq® DNA polymerase (New England Biolabs, USA) were used.

PCR with primer set *Spef-F1* / *Spef-R* used the following conditions: 30 cycles of denaturation at 94°C for 40 s, annealing at 59°C for 40 s, and extension at 72°C for 60 s. PCR with the *VcatoxR-F* / *VcatoxR-R* primer pair used 30 cycles of denaturation at 94°C for 25 s, annealing at 65°C, for 25 s, and extension at 72°C for 25 s. PCR with primer pairs *Vh200-F* / *Vh200-R* used 30 cycles of denaturation at 94°C for 25 s, annealing at 59°C, for 25 s, and extension at 72°C for 25 s. All initial denaturation and final extension conditions were set at 94°C for 5 min and 72°C for 7 min, respectively.

Multiplex PCR amplification of the selected gene markers

Multiplex PCR was performed using all sets of primers in a reaction mixture (20 µl) containing 1.0 µl of DNA extract (extracted from type strain *V. harveyi*, type strain *V. campbellii*, or the variant *V. harveyi* using Wizard® Genomic DNA Purification Kit), 1x PCR buffer with 1.0 mmol l^{-1} $MgCl_2$, 0.3 mmol l^{-1} nucleotides [with a ratio of 1:6 digoxigenin-labeled dNTPs (Roche Applied Science) to unlabeled dNTPs], 0.5 mmol l^{-1} of each primer set, and 0.03 U μl^{-1} of GoTaq® DNA polymerase (New England Biolabs, USA). The following PCR conditions were used: initial denaturation at 94°C for 5 min, 30 cycles of denaturation, annealing, and extension at 94°C for 40 s, 59°C for 40 s, and 72°C for 60 s, respectively, and a final extension of 72°C for 10 min.

Reverse hybridization

Amplified fragments of the *V. harveyi odc* gene (900 bp), *Vibrio campbellii toxR* gene (245 bp), and a putative *V. harveyi* marker (175 bp) were sequenced prior to use as template in hybridization (ABI 3730XLs DNA sequencer from Applied Biosystems, Macrogen, Korea). DNA amplicons from each single PCR reaction mixture was diluted to 10^{-1}, 10^{-2}, and 10^{-3} and blotted in Hybond™-N+ membrane (Amersham Biosciences, United Kingdom) using the Hoefer PR 648 Slot Blot Manifold (Amersham Pharmacia Biotech, United Kingdom; Figure 1). The membranes were soaked for 10 min in a solution of 0.5 mol l^{-1} NaOH, 1.5 mol l^{-1} NaCl followed by a solution with 1.0 mol l^{-1} Tris, 1.5 mol l^{-1} NaCl. Blots were air dried, subjected to UV-crosslinking using the Bio-Rad GS Gene Linker™ UV Chamber (Program C4, 90 seconds; Bio-Rad Laboratories, USA) and used as template with known gene fragments for hybridization. Whereas the templates in conventional DNA hybridization are usually unknown gene fragments and the probe is a known gene fragment, reverse hybridization in this study made use of membrane blotted templates with known genes.

Blots were processed for hybridization by soaking in hybridization solution (5x SSC, 1% blocking reagent, 0.1% Na-lauryl sarcosyl Na salt, 0.02% SDS) at 68°C, 50 rpm, for 1 h, and hybridization was performed by placing the blots in a mixture containing digoxigenin-labeled multiplex PCR products (10 µl) and 990 µl hybridization solution followed by incubation at 70°C, 50 rpm, for 2 h. The blots were processed for detection using an enzyme-linked immunoassay with anti-digoxigenin-alkaline phosphatase Fab fragments and an NBT/BCIP (nitro-blue tetrazolium chloride / 5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt) substrate following the manufacturer's protocol (Roche Diagnostics GmbH, Germany).

RESULTS

Single PCR profile using selected primers

The three primer pairs (Table 1) used in this study could generate different PCR profiles for type strain *V. harveyi*, type strain *V. campbellii*, and the variant *V. harveyi* (Figure 2). PCR using *Spef-F1* / *Spef-R* primer pair and DNA template from type strain *V. harveyi* (NBRC 15634) and variant *V. harveyi* (SW-9702) generated a 900-bp fragment of the ornithine decarboxylase gene (Figure 2A). PCR using primer pair *Vh200-F* / *Vh200-R* produced the 175-bp fragment of an unknown gene only when using DNA template from type strain *V. harveyi* (Figure 2B). Only the PCR using primer pair *VcatoxR-F* / *VcatoxR-R* and DNA template from type strain *V. campbellii* generated the expected 245-bp *toxR* fragment (Figure 2C) in the agarose gel when using optimized annealing temperature of 65°C. There was no amplification product in PCR using no template negative control (Lane 4 of Figures 2A, 2B, and 2C), verifying that no false positives were obtained.

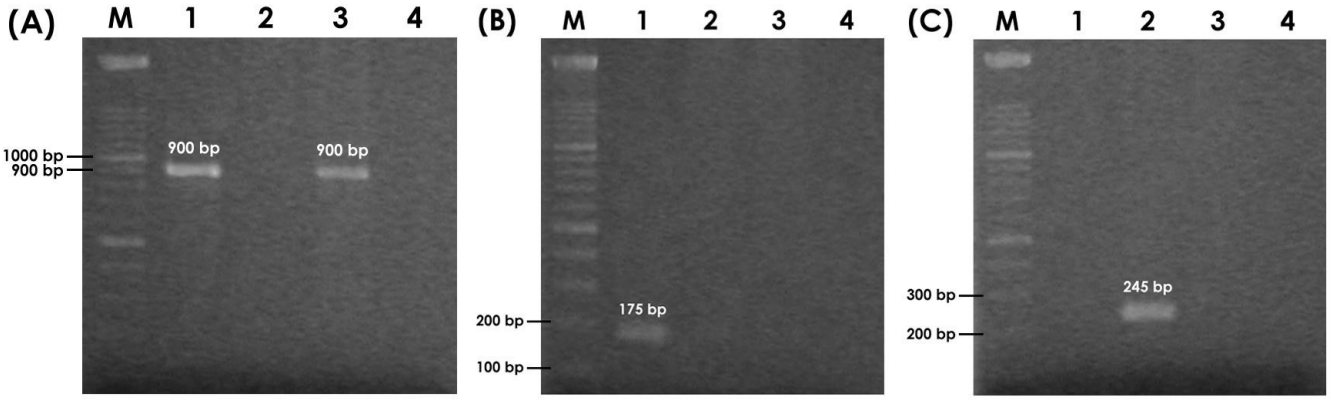

Figure 2. Amplified products of PCR using the selected primers and DNA template from three Vibrios used in this study: A, B, and C, PCR using primer pairs *Spef-F1* / *Spef-R*, *Vh200-F* / *Vh200-R* and *VcatoxR-F* / *VcatoxR-R*, respectively; Lanes 1, 2, 3, and 4, products of PCR using DNA template from type strain *V. harveyi*, type strain *V. campbellii*, variant *V. harveyi*, and no template negative control, respectively; M is the 100-bp ladder (Roche Diagnostics GmbH, Germany).

Multiplex PCR and reverse hybridization profiles

Distinct multiplex PCR profiles that could distinguish the three *Vibrio* isolates used in the study were observed in agarose gels (Figure 3). Multiplex PCR using three sets of primers and DNA template from type strain *V. harveyi* produced three bands of about 900-bp, 245-bp, and 175-bp, representing the expected size amplicons of *odc*, *toxR* and the putative gene marker, respectively (Figure 3A, lane 1). A single 245-bp fragment was obtained in multiplex PCR using type strain *V. campbellii* DNA template (Figure 3A, lane 2) while two bands of about 900-bp and 245-bp were noted in the multiplex PCR profile when using variant *V. harveyi* template (Figure 3A, lane 3). The multiplex PCR amplification profiles of type strain and variant *V. harveyi,* when using the primers for *toxR,* included the 245-bp product because the annealing temperature for the multiplex PCR was lower (59°C) than what was used in single PCR (65°C).

Multiplex PCR was conducted using digoxigenin-labeled dNTPs so that the PCR products could be used as probe in hybridization to confirm if the amplicons are indeed homologous to the blotted known genes, *odc*, *toxR*, and the *V. harveyi* putative gene marker. At 70°C, strong hybridization signals were observed between the dot-blotted 900-bp *odc* and 175-bp putative marker with the multiplex PCR amplicons of type strain *V. harveyi* (Figure 3B, B1). An amplicon from type strain *V. harveyi* hybridized with the 245-bp *V. campbellii toxR* template when the template was not diluted (data not shown). No hybridization signal was observed, however, when the blotted *toxR* DNA template was diluted ten-fold. This is because *toxR* of type strain *V. harveyi* exhibits only 76% sequence similarity with *V. campbellii toxR* (Rañoa and Hedreyda 2005). Strong hybridization signals were observed between the *toxR* template and the multiplex PCR amplicon of type strain *V. campbellii* (Figure 3B, B2) even after ten-fold dilution of hybridization template before blotting. The products of multiplex PCR using the variant *V. harveyi* template exhibited strong hybridization signals with the blotted type strain *V. harveyi odc*. Strong hybridization signal was also observed between *V. campbellii toxR* with amplicons of the variant *V. harveyi* (Figure 3B, B3), but no signal was observed with the 175-bp putative marker. The multiplex PCR profiles in agarose gel and the reverse hybridization profiles of the three *Vibrio* isolates pathogenic to shrimp could be distinguished from one another. The hybridization of the multiplex PCR amplicons and the DNA blotted in the hybridization membrane could be an indirect indicator if target genes are amplified.

DISCUSSION

The availability of primers for the specific detection of gene fragments present in type strain *V. harveyi* and type strain *V. campbellii* paved the way to developing the multiplex PCR- and reverse hybridization-based detection of three *Vibrio* species implicated in shrimp disease. The expected sizes of amplicons (175, 245, and 900 base pairs) from PCR using the available primer sets were distinguishable in multiplex PCR (Figure 3A). Multiplex PCR without reverse hybridization could be used in a differential detection of the three Vibrios. The additional reverse hybridization procedure involves an increase in cost but provides indirect evidence that the correct size amplicons are indeed the target genes without the need to perform amplicon DNA sequence analysis, which entails even higher cost. The hybridization signal could estimate the homology of the amplicon with the target genes. Non-specific, non-target amplicons are expected to register low hybridization signal to no signal, depending on the stringency of hybridization used.

Amplification of the 900-bp *odc* fragment was observed from PCR using *V. harveyi* DNA template but not with *V. campbellii* template, consistent with the previous reports that no

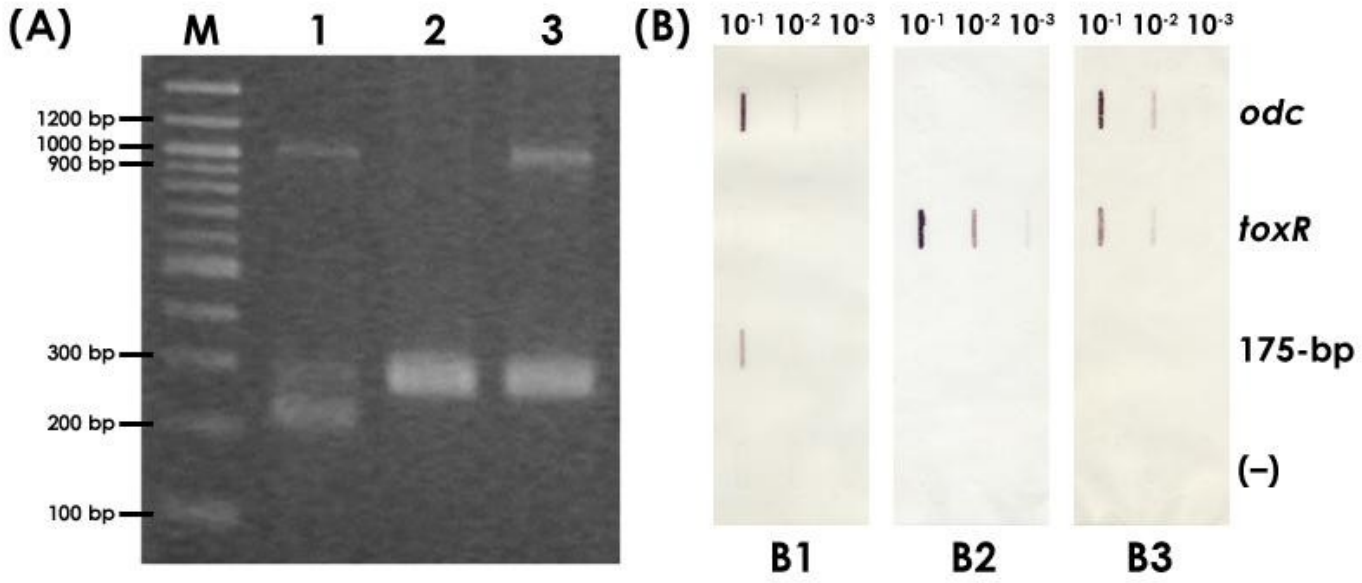

Figure 3. Results of multiplex PCR (A) and reverse hybridization (B): (A) lanes 1, 2, and 3 are products of multiplex PCR using DNA template from *V. harveyi*, *V. campbellii*, and the variant *V. harveyi* strain, respectively; M is the 100-bp ladder (New England Biolabs, USA); (B) B1, B2, and B3, hybridization at 70°C of known DNA fragments with amplicons of multiplex PCR using DNA template from type strain *V. harveyi*, type strain *V. campbellii*, and variant strain of *V. harveyi*, respectively; (–) is the no template negative control.

odc gene has been amplified from *V. campbellii* (Hedreyda and Rañoa 2007). The *odc* gene-targeted primers were crucial in distinguishing *V. harveyi* and *V. campbellii*. The result is also consistent with the observation that *V. harveyi* tests positive for the ornithine decarboxylase assay whereas *V. campbellii* tests negative (Alsina and Blanch 1994; de la Peña et al. 2001). The *odc* gene is present in *V. harveyi* but not in *V. campbellii,* unlike the other genes (*toxR* and gene for hemolysin) used in previous protocols which are present in both, making the detection with *odc* more reliable.

The *toxR*-targeted primers, that were designed based on *toxR* gene from type strain *V. campbellii*, produced amplicons in multiplex PCR using DNA template from any of the three *Vibrio* isolates when using an annealing temperature of 59°C. The *toxR* gene is present in all strains used but the percent similarity of type strain *V. campbellii toxR* with type strain *V. harveyi toxR* is only 76% (Rañoa and Hedreyda 2005), lower than the percent similarity of 92-93% with *toxR* from variant *V. harveyi* (Rañoa and Hedreyda 2005). The band intensity of 245-bp *toxR* amplicons in the agarose gel (Figure 3, lanes 2-3) from type strain *V. campbellii* and the variant *V. harveyi* appeared very similar. When both were allowed to hybridize with the blotted DNA template containing the same concentration of 245-bp *toxR* DNA, stronger signal was observed with type *strain V. campbellii*. This result is consistent with the fact that *toxR* of type strain *V. campbellii* exhibits 100% sequence similarity to the template in the blot and the blotted template only has 92-93% sequence similarity with the variant *V. harveyi*. *toxR* amplicon.

The putative 175-bp *V. harveyi* gene marker that could only be amplified from type strain *V. harveyi* was reported in an earlier study (Conejero and Hedreyda 2003) Detection of this putative marker is very important in distinguishing type strain *V. harveyi* from the other two Vibrios. The 175-bp fragment is not homologous to any known *V. harveyi* gene but could be a non-coding region that is specific for the type strain *V. harveyi.*

Primers used in this study were designed and tested in earlier studies showing that the *odc* and *toxR* gene amplicons could be obtained (but not the unknown 175-bp marker) in PCR using DNA template from all 10 isolates classified as variant *V. harveyi* (Ranoa and Hedreyda, 2007, Castroverde et al, 2006, and Conejero and Hedreyda, 2003). Thus, only one representative isolate, SW-9702, from the variant *V. harveyi* strain was used in the study.

CONCLUSION

A PCR- and reverse hybridization-based protocol has been developed that could distinguish type strain *Vibrio harveyi*, type strain *Vibrio campbellii*, and the variant strain of *V. harveyi* from the Philippines that are pathogenic to shrimp. It is expected that the protocol could detect environmental strains exhibiting characteristics of the variant *V. harveyi.* In this protocol, the detection is not only based on the presence of the correct size of

the amplicon and PCR profile, but, more importantly, based on DNA-DNA hybridization which gives an indirect proof that the amplicons are indeed the target gene markers. This detection protocol could be valuable in detecting the presence of pathogens in ponds as well as in starter cultures of shrimp before seeding in order to avoid disease outbreak and consequent economic loss for shrimp farmers.

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CONFLICT OF INTEREST STATEMENT

There is no conflict of interest among authors and institutions and individuals mentioned above in the conduct of this study and the preparation and submission of this manuscript.

CONTRIBUTIONS OF INDIVIDUAL AUTHORS

Fabini D. Orata conducted the PCR and hybridization experiments under the guidance of Dr. Cynthia T. Hedreyda, who mainly conceptualized the detection protocol in this study. Both worked together to optimize the PCR and hybridization protocols for the specific detection of Vibrios pathogenic to shrimp and to write and revise this manuscript.

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