

# Local *Vibrio* Isolates Exhibit Molecular Characteristics Distinct from Reference *V. harveyi* and *V. campbellii* Strains

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## ABSTRACT

Six *Vibrio* isolates identified biochemically as *Vibrio campbellii* from Southeast Asian Fisheries Development Center (SEAFDEC) in Tigbauan, Iloilo, were characterized by 16 rDNA sequence, total protein profile, and DNA profile analyses. Genomic DNA from the isolates were subjected to PCR using four sets of primers targeting gene fragments of *hemolysin* and *toxR* based on sequences from reference *Vibrio harveyi* (IFO15634), *V. campbellii* (IFO1563), and local isolates identified as *V. harveyi*. Total protein profile could not distinguish the isolates from one another and from the reference *V. harveyi* (IFO15634) and *V. campbellii* (IFO15631). Analysis of 16s rDNA sequences revealed high degree of sequence similarity (96% - 99%) of the six local isolates with other *Vibrio* species including *V. campbellii* and *V. parahaemolyticus*, indicating that this analysis will not be useful in resolving their identity. All six isolates exhibited characteristic reference *V. harveyi* PCR profile when a primer set designed to amplify a 308-bp fragment of *hemolysin* gene in that species was used. However, no amplicons were generated for these isolates using primers that amplify *toxR* gene fragments in *V. harveyi*. This suggests that the six isolates were not bonafide *V. harveyi* strains. The isolates also did not exhibit *V. campbellii* characteristics since the primer designed to target the *toxR* gene in *V. campbellii* could not amplify DNA from any of the six isolates, suggesting that they were not bonafide *V. campbellii* strains either. The *toxR* gene from the six isolates could be amplified using a primer based on *toxR* gene sequences from a SEAFDEC isolate previously identified as *V. harveyi* (PN-9801). These data suggest that the six isolates previously identified as *V. campbellii* as well as PN-9801 may be classified in one group separate from bonafide reference *V. harveyi* and reference *V. campbellii* strains, based on the identical results in the molecular analyses performed in this study.

*Keywords:* *Vibrio*, *toxR*, hemolysin, protein profile, PCR

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## INTRODUCTION

Penaeid shrimp farming, especially of the giant tiger prawn, *Penaeus monodon* dominates crustacean production not only in the Philippines but also in the entire Southeast Asian region. In fact in 1995, a production of 0.48 million metric tons of black tiger prawn was recorded from Southeast Asia (Subasinghe et al., 1997). In the Philippines, production from tiger prawn totaled 36,798 metric tons, accounting for 20% of total yield in 1998 (Philippine Fisheries Profile–BFAR, 1998). One major concern in Philippine shrimp farming industry however, is the emergence of diseases, which results to significant reduction in production.

Diseases in cultured shrimps, which have been causing great production loss in Philippine aquaculture industry, are attributed mainly to bacterial pathogens *V. harveyi* and *V. campbellii*. These pathogens were shown to be among the most dominant species associated with mortalities in black tiger shrimp in the Philippines (de la Peña et al., 2001). Biochemical tests were used to identify and classify the pathogens after heavy mortalities due to luminescent vibriosis have been observed among pond-cultured shrimps. In the same study (de la Peña et al., 2001), it was also demonstrated that several strains, including the six isolates used in this study, were pathogenic when injected intramuscularly to shrimps. However, identity of the local isolates should be confirmed because biochemical tests were not sufficient to distinguish the local isolates from each other or from reference strain *V. harveyi* and *V. campbellii*. The use of 16S rDNA sequences may not be suitable for the purpose, since these sequences in closely related species such as *V. harveyi* and *V. campbellii* have high homologies (about 96% to 99%). In this study, some molecular approaches were performed to characterize and confirm the identity of the six previously mentioned local isolates, identified as *V. campbellii*. The study was conducted with the following objectives:

- (1) To compare total protein profiles of the six SEAFDEC local isolates with those of reference *V. campbellii* and *V. harveyi* strains.
- (2) Evaluate if indeed 16s rDNA sequences will not be able to resolve their identity.

- (3) To compare PCR profiles of the six local isolates with those of reference *V. harveyi* and reference *V. campbellii* strains using four sets of primers namely *Vhemo* which targets *hemolysin* gene fragments of *V. harveyi*, *VhtoxR* and *VctoxR* which amplifies *toxR* gene fragments of reference *V. harveyi* and *V. campbellii*, respectively, and *PNtoxR* designed to amplify *toxR* gene fragments of local isolates previously identified as *V. harveyi*.

## MATERIALS AND METHODS

### Bacterial isolates used

The six local pathogenic *Vibrio* isolates, which this study aims to characterize, were obtained from Southeast Asian Fisheries Development Center (SEAFDEC) in Tigbauan, Iloilo (Table 1). This research also utilized two reference strains: *V. harveyi* (IFO 15634) and *V. campbellii* (IFO 15631), both from the Institute of Fermentation Osaka (IFO) Japan. Another local isolate (PN-9801) identified as *V. harveyi* based on biochemical tests was also used in this study.

Table 1. Local isolates used in this study.

Designation	ID number	Biochemical identification (de la Peña et al., 2001)
Vc 1	CRW-9807	<i>V. campbellii</i>
Vc 2	PIZ-9824	<i>V. campbellii</i>
Vc 3	NRW-9805	<i>V. campbellii</i>
Vc 4	BR-9807	<i>V. campbellii</i>
Vc 5	BRW-9804	<i>V. campbellii</i>
Vc 6	CRWP-9805	<i>V. campbellii</i>

*Vibrio* cultures were continuously maintained at 30°C incubation in Nutrient agar media containing 1.5% NaCl.

### DNA extraction

Genomic DNA from all *Vibrio* isolates used was extracted using the Nucleospin DNA extraction kit commercially available from Clontech Laboratories, Inc. (Palo Alto, CA, USA).

### 16s rDNA sequence analysis

The 16s rDNA fragments of the six isolates were amplified using specific primers targeting this gene. The PCR products were gel-purified and sequenced. Sequence homology of the isolates was then compared with known 16s rDNA sequences of various species deposited in the database using Blast sequence homology search, to determine the degree of relatedness of the isolates with the reference strains.

### Extraction of total proteins

Total proteins were extracted from the six local *Vibrio* isolates, two reference strains: *V. harveyi* and *V. campbellii* and a local isolate (PN-9801) identified as *V. harveyi*. Cells were collected from a 10 ml overnight bacterial culture in liquid broth by centrifugation at 10,000 x g for 2 minutes at room temperature. The resulting pellet was resuspended in sterile distilled deionized water after which 200 mL of 2X Treatment buffer was added. The treatment buffer consist of 0.125M Tris-Cl, 4% sodium dodecyl sulfate (SDS), 20% glycerol, 0.2M dithiothreitol (DTT) and 0.02% bromphenol blue. The cells in treatment buffer were boiled for 10 minutes and then centrifuged at 10,000 x g for 5 minutes at room temperature to pellet the debris and insoluble materials. The supernatant containing total protein extracts were transferred to fresh 1.5-ml tubes. The concentrations of the extracted proteins were determined by spectrophotometry at a wavelength of 280 nm (Ausubel, 1997). Bovine serum albumin (BSA) was used as protein standard. Sample concentration was standardized using 1X TB.

### SDS-polyacrylamide gel electrophoresis (PAGE) of total proteins from the isolates

Total protein extracts of each isolate were run in a 7.5% acrylamide gel using the discontinuous Laemmli SDS-PAGE method. This was done in a Hoefer SE400 Sturdier Vertical Unit [Amersham] setup with 1.5 mm gel spacers. For each sample, 100 mg protein extract was loaded onto each well. Along with the samples, a broad-range protein ladder from Biorad served as molecular weight marker. Proteins run in the gels were

stained using the Vorum Silver Staining protocol (Mortz et al., 2001).

### PCR-based characterization of *Vibrio* isolates

Four sets of primers namely *Vhemo*, *VhtoxR*, *VctoxR* and *PNtoxR*, which were designed in earlier studies to target specific regions of the *hemolysin* and *toxR* genes of reference *V. harveyi* and *V. campbellii* strains as well as two local *Vibrio* isolates, were used in this study. *Vhemo*, *VhtoxR* and *PNtoxR* primers were designed by Conejero (2002) for the specific detection of different strains of *V. harveyi*. The *VctoxR* primer set was designed to amplify a fragment of *toxR* from *V. campbellii* (Sobrepeña et al., 2002). Sequences of each primer set used appear in Table 2.

#### PCR using *hemolysin* primer *Vhemo*

DNA extracted from the isolates and reference strains, *V. harveyi* and *V. campbellii* were used as templates for PCR with primers originally designed for a hemolysin gene fragment. The *Vhemo* primer set targets a unique 308-bp region in the *hemolysin* gene of the reference *V. harveyi* (IFO 15634) and a local *Vibrio harveyi* strain (PN-9801). DNA samples were subjected to PCR in Perkin Elmer Gene Amp System (Perkin Elmer Corporation, Singapore). Conditions for PCR with *Vhemo* primers were as follows: 30 cycles at 94°C for 1 min, 53°C for 1 min, and 72°C for 1 min with an initial denaturation at 94°C for 5 minutes and a final extension step at 72°C for 7 minutes.

Table 2. PCR primer sequences used in the study (Conejero & Hedreyda, 2003).

Primer		Sequence 5' – 3'
<i>Vhemo</i>	Forward	TCA GTGCCT CTC AAG TAA GA
	Reverse	GCT TGA TAA CAC TTT GCG GT
<i>Vhtoxr</i>	Forward	TTC TGA AGC AGC ACT CAC
	Reverse	TCG ACT GGT GAA GAC TCA
<i>Vctoxr</i>	Forward	TTC TGA AGC AGC ACT CAC
	Reverse	TTC TGA AGC AGC ACT CAC
<i>PNtoxR</i>	Forward	AGC AGC TGC TCC AGT TGA
	Reverse	CTG CTC AAT TGA TGG CAG

### PCR using *VhtoxR* primer

*VhtoxR* primers were used to amplify genes from the six local isolates and from reference strains, *V. harveyi* and *V. campbellii*. The primer was based on partial *toxR* gene sequence of a reference *V. harveyi* strain (IFO 15634). Conditions for PCR with *VhtoxR* primers were as follows: 30 cycles at 94°C for 1 min, 63°C for 1 min, and 72°C for 1 min with an initial denaturation at 94°C for 5 minutes and a final extension step at 72°C for 7 minutes.

### PCR using the *VctoxR* primer

*VctoxR* primer set was previously designed to amplify a 230-bp fragment of the *toxR* from *V. campbellii*. This primer set was used in PCR using DNA templates from the six isolates and from reference strains, *V. campbellii* and *V. harveyi*. PCR of the samples with *VctoxR* was accomplished using the following conditions: 30 cycles of denaturation at 94°C for 1 min, annealing at 57°C for 1 min, and extension at 72°C for 1 min with an initial denaturation at 94°C for 5 min and a final extension step at 72°C for 7 min.

### PCR using the *PNtoxR* primers

*PNtoxR* primers were based on partial *toxR* sequence of a local *Vibrio* isolate (PN-9801) previously identified as *V. harveyi*. The primer set was designed to target the 226-bp fragment of *toxR* from local *Vibrio* isolates. DNA from the six local isolates, reference *V. campbellii* (IFO-15631) and a local isolate (PN-9801) were used as template in the following run: 30 cycles at 92°C for 45 secs, 63°C for 1 min, and 72°C for 1.5 min with an initial denaturation at 94°C for 5 min and a final extension step at 72°C for 7 min.

The PCR mix without the DNA template was used as a negative control for all runs. The components for each 20-mL reaction mix are as follows: 13.9 mL sterile distilled water, 2 mL buffer, 0.6 mL MgCl<sub>2</sub>, 0.4 mL dNTPs, 1 mL forward and 1 mL reverse 10mM primers, 0.1 mL *Taq* polymerase, and 1 mL template. PCR products along with a 50-bp or 100-bp molecular weight marker were run in 1.5% agarose gels, stained with ethidium bromide and visualized under ultraviolet light.

## RESULTS AND DISCUSSION

The six *Vibrio* isolates used in this study (CRW-9807, PIZ-9824, NRW-9805, BR-9807, BRW-9804, CRWP-9805) were previously identified by traditional biochemical tests as *V. campbellii* (de la Peña et al., 2001). However, variable responses to biochemical tests are often observed in *Vibrio* species. These tests are also seldom adequate to distinguish between *Vibrio* species especially closely related ones. In a species characterization study of de la Peña and co-workers (2001) for example, only the ability to decarboxylate ornithine differentiates *V. harveyi* from *V. campbellii*. Similar characteristics were exhibited by both strains in all other reactions tested. So to confirm whether the six local SEAFDEC isolates are strains of bonafide *V. campbellii*, molecular tests were performed. A molecular approach offers the speed and ease of method without compromising accuracy of identification. The isolates can be characterized based on their 16s rDNA sequences as well as protein and DNA profiles using powerful molecular tools such as SDS-PAGE and gene-targeted PCR for discrimination between various strains.

### Characterization using total protein profile

The isolates were first characterized based on the protein profiles generated after resolution of total soluble protein in an SDS-PAGE. Using protein profile analysis, relationships between bacteria can be determined based on the presence of unique and shared bands. This was demonstrated by Que (2002) to deduce genetic relatedness in several *Vibrio* reference strains where several protein bands were found to be characteristic of each species. Isolates were therefore expected to exhibit distinct protein profiles that could identify them with any of the reference strains used based on the presence of similar protein bands. Surprisingly, similar total protein profiles were generated by the six isolates (Fig.1), the two reference strains, *V. harveyi* (IFO 15634) and *V. campbellii* (IFO 15634) and a local isolate identified as *V. harveyi* (PN-9801). All were characterized by the presence of two heavily stained bands near the 45 kDa marker and four lightly stained high molecular weight bands between the 97.4 and 66 kDa markers. Compared to the rest,

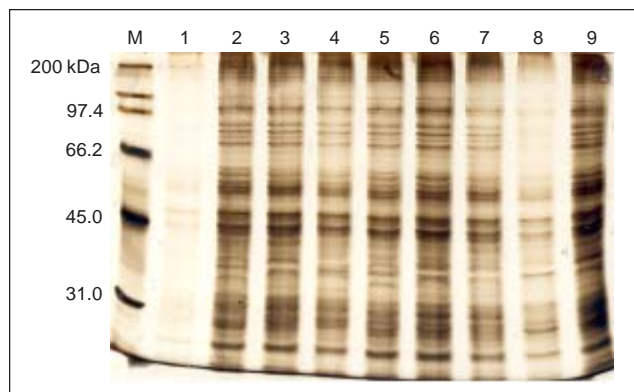


Fig. 1. Total protein profile of the *Vibrio* isolates. Lanes 1-6: CRW-9807, PIZ-9824, NRW-9805, BR-9807, BRW-9804, CRWP-9805; lanes 7 and 8 are reference strains *V. harveyi* (IFO15634) and *V. campbellii* (IFO15631), respectively; lane 9 is a local isolate (PN-9801) identified as *V. harveyi*. M is a broad-range protein ladder from Biorad.

one slight difference though, can be observed with the profile of *V. harveyi*, which is the presence of a distinct band just below the 31 kDa marker. These observed similarities in total protein profiles indicate the close relatedness of the six local isolates with *V. harveyi* and *V. campbellii* reference strains and with a locally isolated *V. harveyi* strain. Protein profile analysis was not able to sufficiently distinguish the isolates from each other nor identify them with any of the two reference strains used.

### Analysis of 16s rDNA sequence

Another way to elucidate genetic relatedness among bacteria is the use of 16s rDNA sequence analysis. Dorsch and co-researchers (1992) have shown the close relationship of *V. alginolyticus*, *V. campbellii*, *V. harveyi*, *V. proteolyticus*, *V. parahaemolyticus*, and *V. natriegens* by analyzing conserved and variable sequences in this gene. An available database containing

known 16s rDNA sequences of various bacterial species allow alignment of new sequences with those already recorded in the database. The use of Blast sequence homology search to analyze newly obtained 16s rDNA sequences of the six local isolates [GenBank Accession Nos. DQ017901 (CRW-9807), DQ017902 (PIZ-9824), DQ017903 (NRW-9805), DQ017904 (BR-9807), DQ017905 (BRW-9804), and DQ017906 (CRWP-9805)] revealed high degree of sequence similarity (96%-99%) in 16s rDNA of the six local isolates with other *Vibrio* species including *V. campbellii* and *V. parahaemolyticus* (Table 3). The high interspecies homology observed in 16s rDNA was also confirmed by phylogenetic analysis using the MEGA v2.1 software (Fig. 2). These observations suggest the need for other molecular typing methods that target specific gene sequences in *Vibrio* species for more accurate classification.

### PCR targeting hemolysin gene fragments

The *hemolysin* gene, found to be present in several *Vibrio* species codes for an important virulence factor in bacteria. Pathogenic activity of hemolysins includes erythrocyte disruption in various species and toxicity for cells and small experimental animals (Nishibuchi & Kaper, 1995). To detect the presence of *hemolysin* gene and to test if primers designed based on *V. harveyi* would work with the six local isolates, *Vhemo* primers were used in PCR. These primers were shown to generate a characteristic 308-bp amplicon from *V. harveyi* reference strains as well as from a variant local isolate (PN-9801) identified as *V. harveyi* (Conejero & Hedreyda, 2004). Amplification of the 308-bp *hemolysin* gene fragment using DNA templates from the five of six local isolates was observed (Fig. 3), indicating that a gene homologue is present in the five local isolates and the sequences in the primer annealing

Table 3. 16S rDNA sequence analysis.

Isolate ID number	16S rDNA sequence similarity
CRW-9807	98% <i>V. campbellii</i> , <i>Vibrio</i> sp, <i>V. parahaemolyticus</i>
PIZ-9824	96% <i>V. campbellii</i> , <i>Vibrio</i> sp, <i>V. parahaemolyticus</i>
NRW-9805	99% <i>V. campbellii</i> , <i>Vibrio</i> sp, <i>V. parahaemolyticus</i>
BR-9807	99% <i>V. campbellii</i> , <i>Vibrio</i> sp, <i>V. parahaemolyticus</i>
BRW-9804	99% <i>V. parahaemolyticus</i> , 98% <i>V. campbellii</i>
CRWP-9805	99% <i>V. campbellii</i> , <i>V. parahaemolyticus</i>

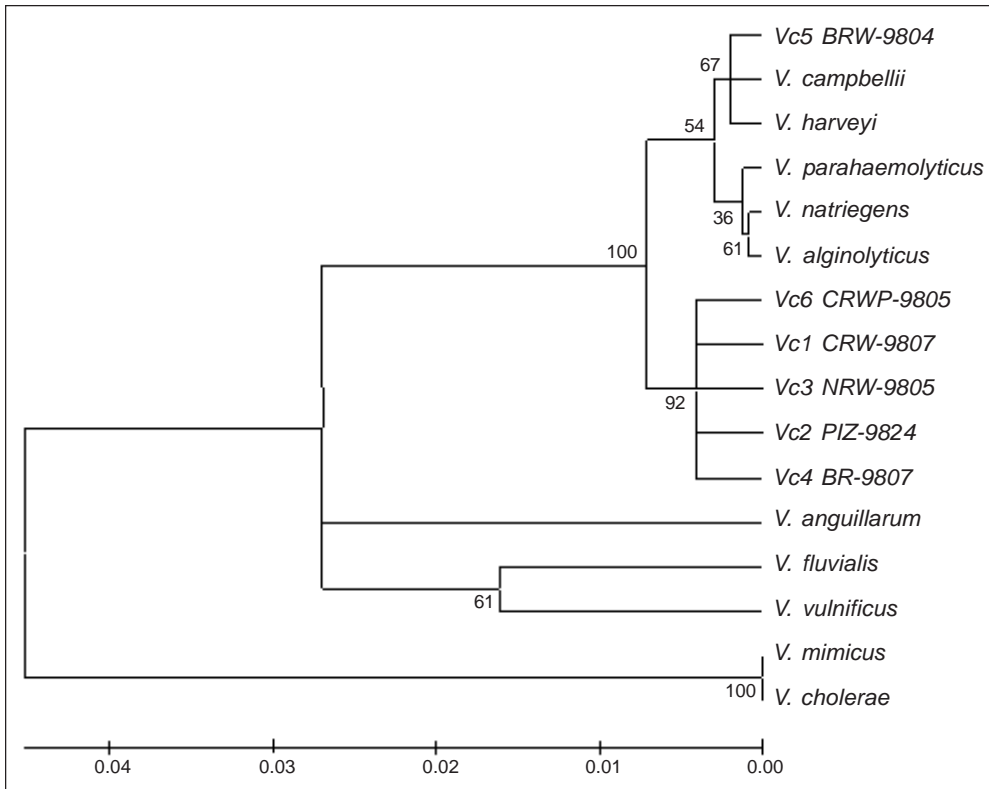


Fig. 2. Unrooted tree constructed by the neighbor-joining method based on the 16s rRNA gene. Percent bootstrap values from 1,000 replicates generated are given at the branching points. Bars indicate genetic distance.

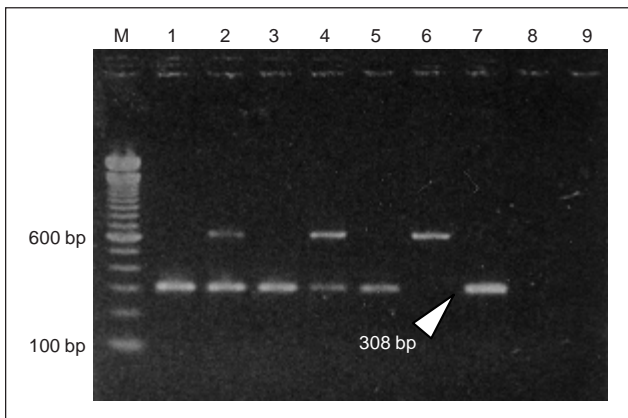


Fig. 3. PCR profile of the six *Vibrio* isolates using *Vhemo* primers. Lanes 1-6 contains the local isolates: CRW-9807, PIZ-9824, NRW-9805, BR-9807, BRW-9804, CRWP-9805; lanes 7 and 8 are products from the reference strains *V. harveyi* (IFO 15634) and *V. campbellii* (IFO 15631), respectively; lane 9 is the negative control (no template). M is the 100-bp molecular weight marker.

sites were significantly homologous to that of the *V. harveyi* reference strain. At 53°C annealing temperature, five local isolates were able to produce distinct 308-bp amplicons with the *Vc2* and *Vc4* (PIZ-9824 and BR-9807, respectively) both exhibiting an

additional band of about 600-bp in their profiles which could not be explained at this point. One isolate, *Vc6* (CRWP-9805), gave only a 600-bp product instead of the expected 308-bp fragment.

The absence of the 308-bp band in the profile of reference strain *V. campbellii* indicates that hemolysin gene sequence variation in the primer annealing sites are present in *V. campbellii*. Data also suggest that *hemolysin* gene sequences of the six local isolates being studied maybe exhibiting greater homology with reference strain *V. harveyi* compared to the *hemolysin* sequences of *V. campbellii*. This adds to the confusion in species confirmation of these local isolates. Although they were previously identified by biochemical means as *V. campbellii*, these results show that they are not exhibiting bonafide *V. campbellii* characteristics.

### PCR targeting *toxR* gene fragments

#### *VhtoxR* primers

The *toxR* gene is considered a main regulator for coordinating expression of various virulence genes in

bacteria. It is also hypothesized to be part of the conserved genes in the ancestral chromosome of members of the family *Vibrionaceae* (Osorio et al., 2000). Sequencing of the *toxR* gene revealed the presence of variable sequences that can be utilized for the identification of specific species of *Vibrio*. A *toxR* homologue from a reference strain *V. harveyi* (IFO 15634) was demonstrated (Conejero, 2002) to be useful for designing primers (*VhtoxR*) specific for *V. harveyi* reference strains. Based on this homologue another primer *PNtoxR* was designed for the detection of *toxR* fragments in locally derived *Vibrio* isolates pathogenic to *P. monodon*. Another set of primers (*VctoxR*) targeting a *toxR* gene fragment in *V. campbellii* was designed (Sobrepeña et al., 2002) based on the partial *toxR* sequence of a reference *V. campbellii* strain (IFO 15631). All the above-mentioned primers were used to test which primer will result in amplification of the *toxR* gene fragment and consequently to test if indeed they will exhibit the characteristic *V. campbellii* results, that is producing amplicons only when using the primers *VctoxR* designed based on *V. campbellii* sequences.

PCR using DNA templates from the six local isolates with *VhtoxR* primers did not result in the amplification of the 390-bp *toxR* fragment that is produced in PCR using *V. harveyi* reference strain DNA template. Although the *Vhemo* primer generated the unexpected 308-bp amplicons for the isolates previously identified as *V. campbellii*, these six isolates did not exhibit profiles characteristic of *V. harveyi* reference strain in PCR using the *VhtoxR* primers. This further complicates the analysis because the use of two different primer set in PCR resulted in conflicting result, one suggesting closer relatedness to *V. harveyi* and the other does not.

#### *VctoxR* primers

When the DNA from six local isolates were subjected to PCR using *VctoxR* primers, designed based on sequences of *toxR* gene fragments of reference *V. campbellii*, no amplicons were observed either (data not shown). Interestingly, data reveals that the *toxR* gene fragment in the six local isolates may have significant sequence variation particularly at the primer annealing sites from those of reference strains *V. campbellii*.

Therefore, the six isolates are not exhibiting bonafide *V. campbellii* characteristics. These findings point out to the possibility that the local isolates could be very close relatives of *Vibrio harveyi* or *Vibrio campbellii* but may later be classified as an entirely different group separate from these two species.

#### *PNtoxR* primers

Another set of primers (*PNtoxR*) based on partial sequences of the *toxR* gene isolated from a local isolate PN-9801, previously identified as *V. harveyi*, could generate a 226-bp band in PCR using DNA template from that local isolate (PN-9801). When DNA from the six isolates used in this study were subjected to PCR using *PNtoxR* primers, the 226-bp amplicon was also produced in all six samples (Fig. 4). No amplicon was produced in PCR using DNA templates from reference *V. harveyi* and *V. campbellii*. These data strongly support the hypothesis that the local isolates previously identified as *V. harveyi* and the local isolates used in this study previously identified as *V. campbellii*, could be belonging to the same classification. Preliminary total protein profile analysis of these local isolates revealed almost identical profiles. The *hemolysin* gene fragment from these isolates could not be amplified by three primers used to amplify *hemolysin* gene fragments from bonafide *V. harveyi* isolates but the primer set *Vhemo* that was able to produce a 308-bp amplicon in reference strain *V.*

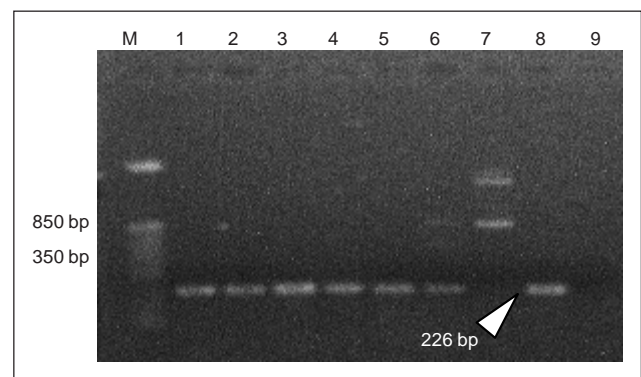


Fig. 4. PCR profile of the six *Vibrio* isolates using *PNtoxR* primers. Lanes 1-6 contains the six local isolates: CRW-9807, PIZ-9824, NRW-9805, BR-9807, BRW-9804, CRWP-9805; lanes 7 and 8 are products from the reference strains *V. campbellii* (IFO 15631) and a local isolate PN-9801, respectively; lane 9 is the negative control (no template). M is the 50-bp molecular weight marker.

*harveyi* as well as the all the local isolates in this study. DNA templates from 6 local isolates in this study, could not be amplified by the *VhtoxR* and the *VctoxR* primers that are used to amplify *toxR* gene fragments from *V. harveyi* and *V. campbellii*, respectively. The molecular data from this study provide preliminary evidence for a proposed separate classification of a local *Vibrio* isolates from previous studies and from this present study, distinct and separate from bonafide *V. harveyi* and *V. campbellii*. where they are currently included based on biochemical tests. One approach to resolve this classification problem involves DNA sequence analysis of additional genes that are present in all *Vibrios* in order to provide additional data for grouping isolates together or discriminating members of different but closely related *Vibrio* species. Candidate gene markers that are being considered include the *gyrase B* gene, *toxR* gene, and the gene for hemolysin (Conejero & Hedreyda, 2003; Conejero & Hedreyda, 2004).

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