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Conventional PCR assays for the detection of pathogenic *Vibrio* spp. in shrimp aquaculture in the Philippines

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Abstract. Shrimp culture is considered as one of the major industries in most aquaculture-producing countries in Asia. This industry has grown steadily over the years, but its gradual decline is due to several disease outbreaks of either bacterial or viral in origin. Luminous vibriosis, which is caused mainly by *Vibrio harveyi* has been implicated for mass mortalities in shrimp aquaculture in the Philippines. In addition, another pathogen, *V. cambellii* also causes vibriosis in shrimp, but this bacterium does not exhibit luminescence. The use of molecular methods particularly the polymerase chain reaction (PCR), facilitated early detection of the causative agent; thus, control measures have been undertaken to ensure efficient management strategies, thus preventing massive mortalities of the cultured stock. This paper reviews the different PCR protocols that have been developed for early detection of pathogenic *Vibrio* spp. in Philippine shrimp aquaculture. These PCR protocols were developed by targeting specific genes of the pathogen in order to ensure accurate diagnosis of the disease. In addition, the use of direct colony PCR, multiplex PCR and co-amplification together with another viral pathogen is also discussed.
Key Words: PCR, luminous vibriosis, *Vibrio* spp., shrimp, aquaculture.

Introduction. Bacteria of the genus *Vibrio* are Gram-negative rod-shaped bacteria that are mainly facultative anaerobic, catalase- and oxidase-positive, motile by polar flagella and mostly require sodium chloride (Farmer et al 2005). These organism are widespread in the coastal and estuarine environments, either free-living or in association with phyto- and zooplankton (Oberbeckmann et al 2011). The taxa of Vibrios that are causing diseases in aquatic animals are increasing, whereas *Vibrio cholerae* is a serious pathogen of humans (Farmer et al 2005; Austin & Austin 2007). *V. parahaemolyticus* and *V. vulnificus* cause diseases in both humans and animals (Austin 2010). Among the *Vibrio* species, *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus* are higher risk Vibrios that are involved in zoonoses (Austin 2010), and there is global concern on the prevention and disease control strategies involving these bacteria.

In aquaculture particularly in the farming of shrimps, Vibrios are also sources of a major disease that has resulted in mass mortalities of the cultured stock. Vibriosis, which is the disease caused by some members of *Vibrio* spp., is one of the limiting factors in the success of shrimp culture operations. Although vibriosis is a common bacterial disease among marine organisms, this disease normally occurs during the warm summer months when the water salinities and organic loads are high (Tendencia 2004).

Luminous vibriosis in shrimps due to the infection of *V. harveyi* occurs in both the grow-out ponds and in hatcheries (Lavilla-Pitogo & de la Peña 1998; de la Peña et al 2001; Parvathi et al 2009). *V. harveyi* is the dominant pathogen that causes luminous vibriosis in shrimp ponds and hatcheries (Leano et al 1998; Sung et al 1999), *e.g.*, in the Philippines the incidence of vibriosis due to *V. harveyi* is 65.5% (de la Pena et al 2001), and in India this bacterium can infect hatcheries and grow-out systems causing large scale mortalities (Karunasagar et al 1996; Abraham & Palaniappan 2004).

Another closely related species is *V. campbelli*, which is a non-luminous bacterium that is also widely distributed in the marine environment. Experimental infection of shrimp with a strain of *V. cambellii* also resulted in the progression of vibriosis in the host (de la Peña et al 2001). This is also implicated as the cause of vibriosis in shrimp aquaculture in the Philippines based on molecular analysis of bacterial isolates obtained from different aquaculture sites (Maeda et al 2002). Both *V. harveyi* and *V. campbellii* are almost similar phenotypically and biochemically, such that those isolates previously identified as *V. harveyi* were actually classified as *V. campbellii* based on molecular identification methods (Gomez-Gil et al 2004). Despite of the similarities between these two strains of *Vibrio*, both pathogens cause vibriosis in the cultured shrimp.

The problems brought about by vibriosis in the shrimp culture industry led to the emergence of alternative strategies to prevent disease outbreaks. These include the use of the so-called ”green-water” technology derived from finfish (Huervana et al 2006), probiotics application, use of reservoir and chlorination of ponds during the culture period (Corre et al 2000). In addition to these methods, routine surveillance of the culture sites has to be done to detect the pathogens during the early stages of infection. Luminous Vibrios as well as presumptive Vibrios are monitored using standard microbiological techniques. This involves regular sampling of the rearing water and the cultured stock by plating them on a selective medium, *e.g.* thiosulfate-citrate bile salt agar, TCBS for *Vibrio* spp., that will allow growth of the target pathogen. However, this method may not be able to detect the pathogen during the early stages of infection or it may have a lower sensitivity of detection. As such, other methods particularly molecular-based detection methods such as polymerase chain reaction (PCR) have been developed to provide a fast and accurate determination of the pathogens at the early stages of infection. In this way, effective management procedures could be implemented to prevent severe disease outbreaks and production losses.

Conventional PCR as a diagnostic tool in aquaculture. With the advent of rapid advances in the field of molecular biology, a surge in the growth of new methods for diagnosing fish diseases has also been observed. In particular, the use of the polymerase chain reaction has been developed to ascertain the presence of the viral and bacterial pathogens in fish and shellfish (Austin 1998). The polymerase chain reaction (PCR) is used to amplify certain regions of the DNA. Short oligonucleotide primers are designed that will hybridize to each end of the target region of the DNA to be amplified. The reaction includes template DNA that may be in various forms, *e.g*., tissue lysate to purified DNA, primers, polymerase enzyme that will catalyze the creation of new copies of DNA, and nucleotides to form new copies. In each round of the thermocycling reaction, the template DNA is denatured, primers anneal to their complementary regions and the polymerase enzyme catalyzes the addition of nucleotides to the end of each primer; hence, creating new copies of the target region. Theoretically, the increase in the amount of product after each round is geometric. The presence or absence of a product following PCR may be sufficient to indicate whether the sample is infected by a certain pathogen. However, to provide thorough details on the identity of the original sample used, restriction enzyme digestion, probe hybridization and even nucleotide sequencing of the PCR products are employed (McBeath et al 2000).

PCR diagnosis may greatly increase the speed of diagnosis especially in instances when the pathogens require lengthy culture period prior to identification. The sensitivity of detection is also significantly enhanced when PCR is used directly or in conjunction with other techniques. PCR can be carried out by amplification of a short DNA sequence that is added to an antibody (Cunningham 2002). This antibody is subsequently used in immunosorbent assay with the amplification of the DNA tag, thus improving the sensitivity of detection for the bound antibody (Patel et al 1997).

There are many factors that are needed to be considered in employing PCR for diagnostic purposes. Firstly, the purpose of the PCR should be clearly defined, *i.e*., whether the presence of absence of the PCR product is desired as the final result or whether further processes such as enzyme digestion will be carried out. Primer design is also another factor to consider in order to obtain greatest possible sensitivity and

specificity. Therefore, primers should be sufficiently long to permit high annealing temperature and reduce the chance of nonspecific primer annealing. However, primers that are too long may facilitate nonspecific annealing even to regions of DNA that are not perfectly complementary to the primer sequence (Cunningham 2002).

Template preparation is another critical step in any diagnostic test. Traditional methods of nucleic extraction require the use of harmful chemicals and may take a considerable time to perform. Recently, there are many commercial kits that have been made available to facilitate ease in extraction, but sometimes the use of these kits results to reduced yields and therefore affect the sensitivity of PCR. Lysates or homogenates obtained from fish tissues can be prepared rapidly but may contain substances that can inhibit DNA amplification. In many cases, several works have been undertaken using pure cultures or cloned DNA (Cunningham 2002).

The use of PCR for detection of bacterial pathogens is increasingly important because these organisms are a vital component of both freshwater and marine environments. Early and accurate detection of these pathogens is a crucial factor affecting the success of aquaculture systems (Cunningham 2002) and hatchery operations (Muroga 2001).

Conventional PCR assays have been developed to detect luminous *Vibrio* spp. in various organisms such as fish, shrimp and shellfish (Hernández & Olmos 2004; Pang et al 2006; Thaithongnum et al 2006; Sun et al 2009; Cao et al 2010; Hwang et al 2010). In the Philippines, there are several PCR protocols for the diagnosis of vibriosis in shrimp by targeting gene-specific regions of the etiological agent. As a rule, the choice of the genes for PCR detection of a bacterial pathogen must be species-specific, widely distributed and stable in the bacterial genome (Haldar et al 2010) Table 1 shows the different PCR assays for the detection of pathogenic *Vibrio* spp. in shrimp aquaculture. The succeeding sections discuss in detail the importance of these gene-specific regions for pathogen detection in shrimp.

Table 1

PCR assays for the detection of luminous *Vibrio* spp. in shrimp culture using different gene-specific regions

Gene-specific region	Pathogen	Reference
Ribonuclease P (Rnase P) RNA	Various *Vibrios*	Maeda et al 2002
toxR	*V. harveyi* Various *Vibrios*	Conejero & Hedreyda 2003 Rañoa & Hedreyda 2005
Hemolysin (*vhh*)	*V. harveyi* *V. cambellii*	Conejero & Hedreyda 2004 San Luis & Hedreyda 2006

Ribonuclease P RNA gene. Among bacterial isolates, the 16S rRNA has been used to identify and classify bacteria into their specific groups (Maeda et al 2001). There have been a significant amount of sequence data that were accumulated from different bacteria; thus, enabling to establish phylogenetic relationships among the different bacterial groups. However, the use of 16S rRNA in bacterial classification becomes limited when closely related bacterial species are examined (Stackebrandt & Goebel 1994), and the reasons for these are the existence of multiple copies of the 16S rRNA gene (16S rDNA) in the chromosome or the presence of intrachromosomal differences (Maeda et al 2001). An example of the group of closely related bacteria are those belonging to the *Vibrio* core species including *V. parahaemolyticus*, *V. alginolyticus*, *V. carchariae*, *V. natriegens*, *V. campbellii*, *V. proteolyticus*, *V. pelagius* and *V. harveyi*, in which the interspecies differences in their 16S rDNA sequence range only from 0.1 to 1.9% (Dorsch

et al 1992). When 16S rDNAs were compared among the pathogenic and non-pathogenic Vibrios that were isolated from shrimps and in their rearing ponds in the Philippines, Monsalud et al (2003) could not find any conclusive evidence to classify them into separate clades or to distingush the pathogenic Vibrios from the non-pathogenic isolates. As such, other genetic markers are required to elucidate the relationships of these *Vibrio* core species and to differentiate them from one another.

The ribonuclease P (RNase P) is a ribonucleoprotein nuclease that cleaves tRNA precursors, thus generating the mature 5'-end of the tRNAs. This bacterial enzyme has two sub-units, namely, a RNA consisting of 350-400 nucleotides and a protein of approximately 120 amino acids (Pace & Brown 1995). RNase P is essential for all organisms and this exists as a single copy in the chromosome (Maeda et al 2001). Its sequence is more variable than the 16S rRNA, thus, it can clearly differentiate closely-related bacterial species.

Maeda et al (2001) sequenced the gene encoding 16S rRNA from diverse species of Vibrios. They found that within the gene sequence, there were two variable regions among the different Vibrios. These regions are the P3 and P12 helices. The P3 helix however, is composed of longitudinal repetitions of stem structures and the number of repetitions varied among the *Vibrio* core species. This stem structure consists of tandem repeats of palindromic sequence, 5'-GUUGAUGUCCUUCGGGAGACUGAC-3' (Maeda et al 2001). Figure 1 shows the P3 helix structure of the different *Vibrio* core species. These tandem repeats are variable in the *Vibrio* core species, ranging from one to four stem structures. Both *V. alginolyticus* and *V. proteolyticus* have 1 stem structure. *V. pelagius*, *V. natriegens*, *V. parahaemolyticus*, *V. carchariae* and *V. campbellii* have 3 stem structures, while *V. harveyi* has 4 repeats. As such, this P3 helix can be used to differentiate the isolates and strains of Vibrios.

The P3 helix of the Vibrios core species was further used to develop a PCR assay to differentiate the *Vibrio* populations that are present in shrimps and in the culture ponds at selected sites in the Philippines (Maeda et al 2002). Using P3 helix-specific primers, the amplified fragment lengths of the different *Vibrio* species are: 146 base pairs (bp) in *V. harveyi*, 72 bp in *V.alginolyticus*, 83 bp in *V. proteolyticus*, and 120-121 bp in *V. cambellii*, *V. carchariae*, *V. natriegens*, *V. parahaemolyticus* and *V. pelagius*. When the different presumptive Vibrios were used for the PCR assay, results showed that most of the amplified products consisted of three tandem repeats, which is typical of *V. campbellii*. This likely indicates that during the time of sampling, *V. campbellii* was the dominant species that caused vibriosis in shrimps. It was reported that *V. campbellii* also caused vibriosis in shrimps in the Philippines (de la Peña et al 2001).

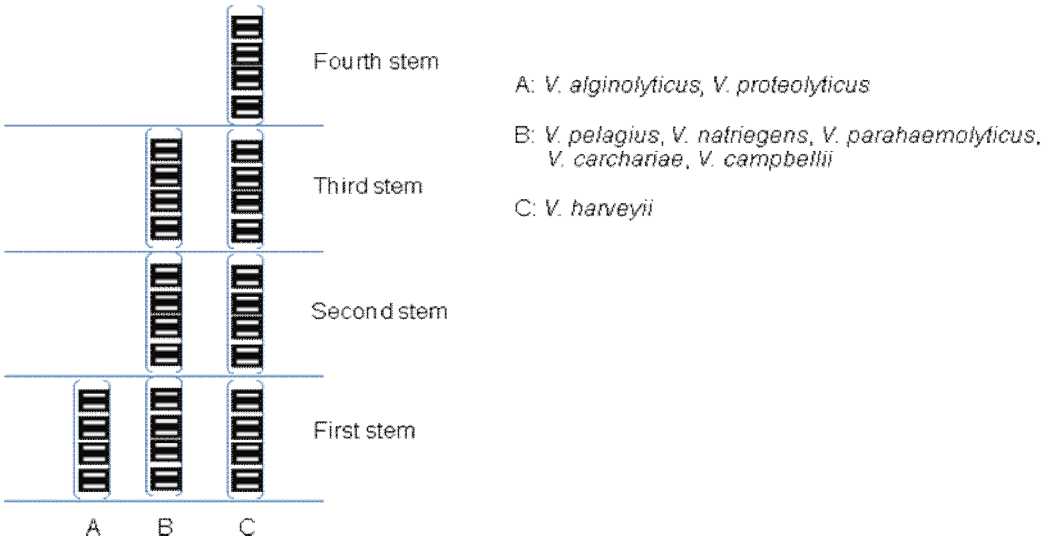

Figure 1. Structure of the P3 helix of the RNase P gene among *Vibrio* spp. This diagram was adapted from Maeda et al (2002).

ToxR gene. The *tox*R gene was first described in *V. cholerae* as a regulatory gene for the cholera toxin and other determinants of virulence (Miller et al 1987). The *tox*R of *V. cholerae* has three functional domains: a cytoplasmic domain, a transmembrane domain and a periplasmic domain. The cytoplasmic domain binds to the heptamer motif of the cholera toxin promoter (Miller et al 1987). On the other hand, the periplasmic domain is important for the interaction with the ToxS protein as well dimerization of the ToxR (DiRita & Mekalanos 1991). ToxR also functions as a regulator of transcription of other proteins, that act as sensors of environmental conditions (Kovacikova & Skorupski 1999; Skorupski & Taylor 1999), as such this gene is important for DNA binding and transcriptional activation in bacteria (Ottermann et al 1992). The *tox*R gene was subsequently found in *V. parahaemolyticus* (Lin et al 1993) in other species of Vibrios (Osorio & Klose 2000). There is heterogeniety within the coding sequence of *tox*R gene, thus, this gene is used to distinguish various *Vibrio* species (Osorio & Klose 2000).

The functional domains of the *tox*R gene from *V. harveyi* is shown in Figure 2. The *tox*R sequence (Genbank accession number DQ503438.1) was obtained from a public database and analyzed of its functional domains using the SMART program (http://smart.embl.de/smart/). It has a transcriptional regulatory domain, which is important for transcriptional activities as well for interaction with the promoter elements of the *tox*R gene. It also has a transmembrane domain, which might have a role in the interaction with other protein elements.

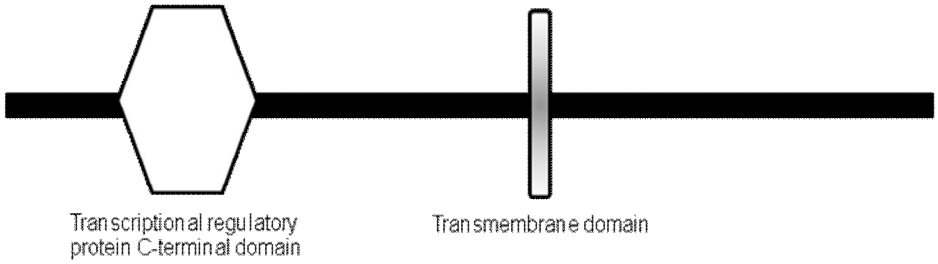

Figure 2. Functional domains of the *tox*R gene in *Vibrio harveyi*.

Among the *Vibrio* isolates obtained from shrimps and in culture ponds in the Philippines, Conejero & Hedreyda (2003) obtained partial fragments of the *tox*R gene from these isolates and designed specific primers for the detection of *V. harveyi*. The primers (forward: 5'-TTCTGAAGCAGCACTCAC-3'; reverse: 5'-TCGACTGGTGAAGACTCA-3') amplified a 390 bo fragment. There were no cross reactions with closely related *Vibrio* species, hence, the primers could be useful to detect *V. harveyi*, which is pathogenic to shrimps. Subsequently, Rañoa & Hedreyda (2005) developed *tox*R-targeted primers (forward: 5'-AGCAGCTGCTCCAGTTGA-3'; reverse: 5'-CTGCTCAATTGATGGCAG-3') to detect Philippine isolates of *Vibrio* spp., which are implicated in vibriosis in shrimps. The primers amplified a 226-bp fragment. These Philippine isolates were related to either *V. harveyi* or *V. campbellii* (de la Peña et al 2001), but did not result in positive amplification using the *tox*R primers developed for the type strains of either *V. harveyi* or *V. campbellii* (Cortado et al 2004). It was suggested that these Philippine isolates exhibit a high degree of sequence variation. Sequencing of the partial *tox*R fragments of these Philippine isolates showed that they were more closely related to *V. campbellii* than with *V. harveyi* (Rañoa & Hedreyda 2005). A complete coding sequence of the *tox*R gene obtained from the type strain of *V. harveyi* revealed that this gene was more divergent than the 16S rRNA, thus it will facilitate clearer distinction between *V. harveyi* and other closely related *Vibrio* species (Franco & Hedreyda 2006).

Hemolysin (*vhh*) gene. Hemolysin is a widely distributed toxin among different populations of Vibrios, and this can cause hemorrhagic speticemia and diarrhea in fish (Thompson et al 2004). This gene is highly conserved within the same *Vibrio* species (Rock & Nelson 2006). It is an exotoxin, which results in the lysis of erythrocytes, thus

releasing iron-binding proteins including hemoglobin, transferrin and lactoferrin. The availability of these iron compounds triggers siderophore production that competes with the iron acquisition system of the host (San Luis & Hedreyda 2006). It also exhbited strong phospholipase activities and was cytotoxic to gill cells in some fish species (Zhang et al 2001). Other cells that are lysed by the exotoxin include neutrophils, mast cells and polymorphonuclear cells (Zhang & Austin 2005), hence, hemolysin is an important virulence factor among pathogenic Vibrios (San Luis & Hedreyda 2006).

Gene sequences encoding for hemolysins have been identified in different *Vibrio* species including *V. harveyi* (Zhang & Austin 2005; Parvathi et al 2009), *V. campbellii* (Haldar et al 2010), *V. parahaemolyticus* (Nishibuchi & Kaper 1985), *V. mimicus* (Kim et al 1997), *V. vulnificus* (Chang et al 1997), *V. cholerae* (Rader & Murphy 1988) and *V. anguillarum* (Hirono et al 1996). An analysis of the gene structure of hemolysins from *Vibrio* spp., showed they possess the conserved motif of amino acids, Gly-Asp-Ser-Leu, hence, it was proposed that hemolysins belong to the GDSL (Gly-Asp-Ser-Leu) family of proteins (Sun et al 2007). Using the *V. harveyi* hemolysin (Genbank accession number EU862239.1) as a model, the sequence was analyzed for the presence of the different domains by PROSITE (http://www.expasy.org). It revealed the presence of the conserved domain of the GDSL protein family within the gene sequence (Figure 3).

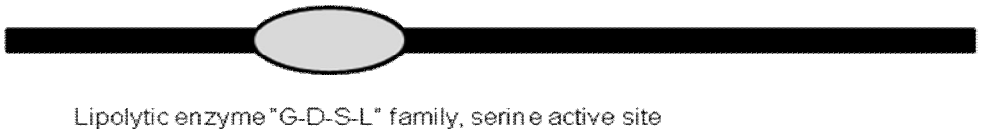

Figure 3. Fuctional domain of the hemolysin gene in *Vibrio harveyi*.

In the Philippines, the hemolysin genes of both *V. harveyi* (Conejero & Hedreyda 2004) and *V. campbellii* (San Luis & Hedreyda 2006) have been identified. PCR primers that amplified a 308-bp fragment of the hemolysin gene were highly specific for the detection of *V. harveyi* isolates not only in the Philippines but also in isolates obtained from Thailand and Ecuador. These primers were a combination of the forward primer (5'-ATCATGAATAAAACTATTACGTTACT-3'; Zhang et al 2001) and reverse primer (5'-GCTTGATAACACTTTGCGGT-3'; Conejero & Hedreyda 2004). Full length sequence of the hemolysin from a type strain of *V. campbellii* was determined (San Luis & Hedreyda 2006). It showed that this gene is highly similar among *V. campbellii* strains but exhibited significant divergence with the hemolysin gene of *V. harveyi*, which is a closely related species.

Direct colony PCR. Amplification of the target gene from bacterial samples was done using whole bacterial cells as template. A common method for this is to boil the bacterial cells in a lysis buffer for few minutes and then use the lysate immediately for PCR (Maeda et al 2002; Pang et al 2006). This step omits the extraction of the DNA and thus considerably saves time in detecting the pathogen. Direct colony PCR was done to detect luminous Vibrios in shrimps using the primers targeting the RNase P gene of *Vibrio* spp. (Caipang & Aguana 2010). Presumptive colonies of *Vibrio* spp. that were obtained from aquaculture ponds and from infected shrimps in selected sites in the Philippines were individually picked using sterile glass tips and immediately placed in tubes containing the PCR premix and the primers for detection. PCR amplification was done following standard thermocycling procedures and the PCR products were electrophoresed in an agarose gel. Results showed that using whole bacterial cells, the target gene could be amplified and thus eliminating the step for DNA extraction. It is recommended that direct colony PCR should be used in routine examination of the cultured stock to determine infection of the bacterial pathogen during the early stages.

Multiplex PCR. Through the use of multiple primers, the simultaneous detection of different bacterial species by conventional PCR is possible, and is known as multiplex

PCR. Multiplex PCR-based detection is a popular and effective method to distinguish closely related bacterial species such as Vibrios (Edwards & Gibbs 1994; Haldar et al 2010). This is carried out either through the use of different gene-specific primers to detect various strains of a particular species of *Vibrio* (*e.g.* Rodkhum et al 2006) or through the use of a single gene-specific primer set to differentiate Vibrios (*e.g.* Haldar et al 2010). Table 2 shows the different multiplex PCR assays that have been developed for the detection of *Vibrio* spp.

Table 2

Multiplex PCR assays on selected *Vibrio* spp.

Target gene	*Vibrio* species	Reference
Hemolysin genes	*V. anguillarum*	Rodkhum et al 2006
Collagenase, *omp*K and *tox*R	Virulent strains of *V. alginolyticus*	Cai et al 2009
Hemolysin gene	*V. harveyi* *V. cambellii* and V. *parahaemolyticus*	Haldar et al 2010
*gyr*B and *pnt*A genes	Human pathogenic and non-pathogenic *Vibrio* spp.	Teh et al 2010
*vvh*A (hemolysin gene)	Strains of *V. vulnificus*	Han & Ge 2010
*tox*R gene	*V. cholerae*, *V. parahaemolyticus* and *V. vulnificus*	Neogi et al 2010
tlh, *tdh* and *fla* genes	Strains of *V. parahaemolyticus*	Wang et al 2011
*rpo*A gene	Strains of *V. cholerae*	Jeyasekaran et al 2011

The different pathogenic Vibrios that infect shrimps in the Philippines were detected simultenously using multpilex PCR by designing different primer sets for the amplification of the hemolysin and *tox*R genes (Castroverde et al 2006). Using primer sets to amplify the hemolysin gene of the Philippine isolates of Vibrios and the *tox*R gene in the type strains of *V. harveyi* and *V. campbellii*, the resulting PCR products when resolved in agarose gels after elecrophoresis showed clear differention of the different *Vibrio* strains. The primers (forward: 5'-GCATTGGGTGACAGCTTGTCG-3'; reverse: 5'-CGGTTGTAGTTCATGAAGTCATTC-3' designed by Castroverde et al (2006) amplified a 320-bp of the hemolysin gene from Philippine isolates of *Vibrio* spp., that were pathogenic to shrimps. The primers (Conejero & Hedreyda 2003) for *tox*R gene of the type strain of *V. harveyi* amplified a fragment of 390-bp. Whereas, the primers (forward: 5'-CCGCTTTCTGCTGACTCTACC-3'; reverse: 5'-GGCTTAGTCAACATCAGTACACAG-3') that amplified a 245-bp fragment of the *tox*R gene in the type strain of *V. campbellii* were designed by Castroverde et al (2006). All the primer sets were specific to the respective genes they amplify at an annealing temperature of 65^{o}C.

Co-amplification with a viral pathogen. Using published primers (forward: 5'-TGACTGGGTAGTCGCTGCTT-3'; reverse: 5'-TATGAAGCCCGGACTTTCCT-3') for the amplification of the Rnase P gene in *Vibrio* spp. (Maeda et al 2002), the presence of

luminous Vibrios was detected together with a viral pathogen, white spot syndrome virus (WSSV) in shrimp (Caipang & Aguana 2010). This duplex PCR was optimized in order for this assay to be applicable in shrimp aquaculture in the Philippines. Both luminous vibriosis and white spot syndrome are serious diseases that affect the shrimp culture industry, and the simultaneous detection of both pathogens could facilitate early diagnosis of the disease.

In this duplex PCR assay, the gene-specific primers for pathogenic Vibrios were able to amplify a target with sizes ranging 120-146 bp. For the detection of WSSV, the gene-specific primers (forward: 5'-ACCTCTTTACTCCCTCGACT-3'; reverse: 5'-TTGTAGAGGGCATGAGGGAT-3', Takahashi et al 1996) were able to amplify a PCR product with a size of 330 bp. The optimized PCR assay conditions resulted in the amplification of both genes as shown by the appearance of PCR bands in the agarose gel during electrophoresis. DNA extracts of shrimps collected from different aquaculture sites were used in the duplex PCR assay and the results also showed the amplification of both genes in some samples, indicating the presence of dual infections in some shrimps. This duplex PCR assay has a potential use for on-site detection of pathogens, however, its level of sensitivity in comparison with conventional PCR has to be properly evaluated.

Conclusions. Pathogenic *Vibrio* spp. cause serious problems in the shrimp aquaculture in the Philippines. During the early stages of infection, the pathogen can be detected by conventional PCR through amplification of specific genes. Several gene-specific primers have been designed to detect pathogenic Vibrios in shrimp, and these should be validated for applications in the field. Regardless of the gene-specific primers that will be used, the pathogen can be detected by conventional PCR in a number of approaches as shown in Figure 4. A shrimp that is suspected to be infected with the pathogen is brought to the laboratory to either isolate bacterial colonies from selected organs or to extract DNA samples from the animal. The bacteria that grow in a selective medium (in the case presumptive Vibrios, these are grown in thiosulfate citrate bile salt agar, TCBS) are processed for the extraction of the genomic DNA. Using gene-specific primers to detect the pathogen, PCR amplification is carried out using either 1) whole bacterial cells, 2) bacterial genomic DNA or 3) DNA samples from infected shrimp. At the end of the reaction, the PCR products are electrophoresed in an agarose gel and visualized for the presence of the band, which corresponds with the size of the amplified product. The appearance of the band on the gel is an indication of the presence of the pathogen in the shrimp or the bacterium that was analyzed is a pathogenic *Vibrio* sp.

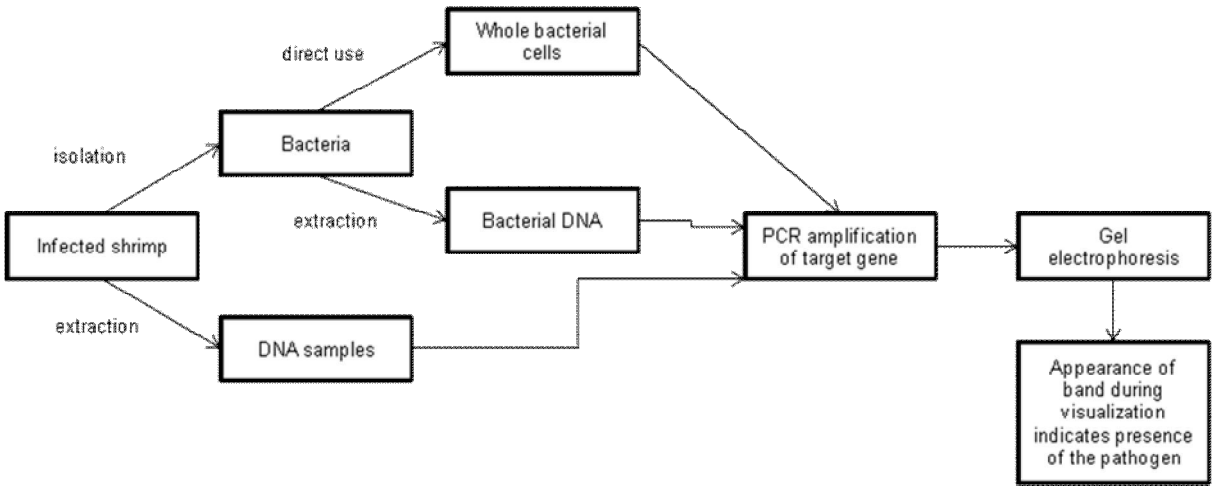

Figure 4. Diagrammatic representation of the PCR protocol for detection of pathogenic *Vibrio* spp. in shrimp.

In conclusion, pathogenic *Vibrio* spp. that infect shrimp aquaculture in the Philippines can be detected using conventional PCR. Routine use of this assay is encouraged as one of the management approaches in disease prevention of the shrimp stock. This molecular approach, in combination with other strategies for effective health management in aquaculture would result in good production through lower incidence of mortalities during the culture period.

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