

## An Overview of PCR Techniques for Shrimp Disease Diagnosis in Asia, with Emphasis on Thailand

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### ABSTRACT

Asia leads the world in cultivated shrimp production with export earnings in the order of billions of US dollars per year. In spite of this success, annual production decreased in the late nineties because of widespread epizootics caused by new viral pathogens. Although, these viruses were no cause for alarm to human health authorities, they were economically crippling for Asian shrimp farmers. In Thailand, shrimp production trends have mirrored those in the rest of Asia, except that recovery from the viral epizootics has been somewhat better than it has for most of its close neighbors. Our work in Thailand has focused on the characterization of the causative viruses and on the development of rapid diagnostic probes for them. Similar work has been done elsewhere. The aim of the work has been to develop effective control measures to help shrimp farmers. We are engaged in similar work on bacteria and parasites. The major viruses of concern (in our estimated order of economic impact for Thailand) are white-spot syndrome virus (WSSV), yellow-head virus (YHV), hepatopancreatic parvovirus (HPV), monodon baculovirus (MBV) and infectious hypodermal and haematopoietic virus (IHHNV). We have also prepared probes for *Vibrio parahaemolyticus* and for a microsporidian parasite, *Agmasoma penaei*. These highly specific and sensitive tools for detection are already helping shrimp farmers and we hope that new technological advances will make them practicable in the field. At the moment, however, the most rapid test is the polymerase chain reaction (PCR) test, which takes approximately 3 hours to complete. This review covers important Asian shrimp diseases for which PCR tests are currently available.

### INTRODUCTION

Asia has always led world production of cultivated shrimp with a market value of billions of US dollars per year. Thailand alone has been the world's leading producer since 1992 with its export earnings alone reaching more than one billion US dollars per year. However, in Thailand in 1995, largely due to yellow-head virus (YHV), production decreased by about 5,000 metric tons (equal to approximately 40 million US dollars in lost export revenue) (Flegel *et al.*, 1995b). In 1996 and 1997, another virus called white-spot syndrome virus (WSSV) was even more disastrous, with cumulative lost export revenue estimated at approximately 1 billion US dollars (Flegel and Alday-Sanz, 1998; Flegel, 1997). After 1997, Thai production began to recover, reaching the previous highest production of 250,000 metric tons again in 1999. The rest of Asia did not fare so well. For example, WSSV outbreaks in China began in 1993, reducing export production from the 1992 high of 115,000 metric tons to 35,000 metric tons. Recovery has been slow, with production reaching only 70,000 metric tons by 1999.

These examples serve to illustrate how serious disease losses can be in the shrimp aquaculture industry. The perilous position of the shrimp farmer and the shrimp industry can be greatly improved by the implementation of relevant strategies which include programs for improved farmer cooperation and technological changes. These strategies could lead to a long term, stable shrimp industry with little negative environmental impact. Biotechnological research can make substantial contributions towards maintaining achieving this goal but it is essential that government and industry provide continuous support for the infrastructure and training required to maintain the relevant capability.

This review covers steps in the development of DNA probes and PCR technology for detection of shrimp pathogens. Much work described has been done in Thailand and it has been reviewed in a broader context elsewhere (Flegel, 1997). Where appropriate, similar work done elsewhere will be included. While focusing on these probes, one should not forget that the probes play only one small part in the overall strategy to fight against disease. They are not an answer in themselves but must be used properly in the overall context of a shrimp health program involving such topics as environmental safety, nutrition, and genetics, to name only three.

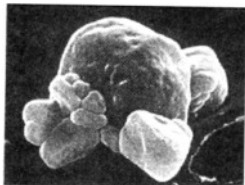
This review will cover the development of DNA diagnostic probes for white-spot syndrome virus (WSSV), yellow-head virus (YHV), hepatopancreatic parvovirus (HPV), monodon baculovirus (MBV) and infectious hypodermal and haemotopoietic parvovirus (IHHNV). Also briefly discussed will be probes developed in Thailand for *Vibrio parahaemolyticus* and for the microsporidian parasite, *Agmasoma penaei*, and probes of others for Taura syndrome virus (TSV), spawner mortality virus (SMV) and a mycoplasma. In terms of losses to the Asian shrimp industry WSSV, YHV, HPV and MBV are undoubtedly the most important (in decreasing order). Losses from the virus IHHNV and from bacteria and microsporidians are less clearly evident.

## MONODON BACULOVIRUS (MBV)

We were quite alarmed when we saw this virus (Figs. 1-3) in Thailand for the first time in 1990 (Fegan *et al.*, 1991), because it had been implicated in the collapse of the shrimp industry in Taiwan in the mid 1980's (Lin, 1989). However, we soon found out that it did not cause shrimp mortality so long as rearing conditions were good. This was in spite of the fact that some of the infected shrimp larvae had very large numbers of viral inclusion bodies. In other words, they survive high levels of virus production with no ill effects and no visible resistance response. The opinion that MBV did not cause mortality was later expressed in Taiwan as well (Liao *et al.*, 1992).

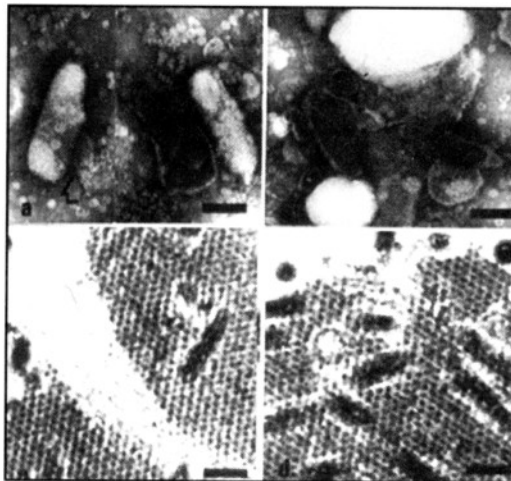


**Figure 1.** Squash mount of a larval hepatopancreatic cell showing several polygonal viral inclusion bodies in the enlarged nucleus. The inclusions are composed of a protein matrix called polyhedrin which contains embedded viral particles. Because these protein particles enclose or protect viral particles, they are sometimes called occlusion bodies. They are released in the shrimp feces and ingested by other larvae so that the infection is spread horizontally



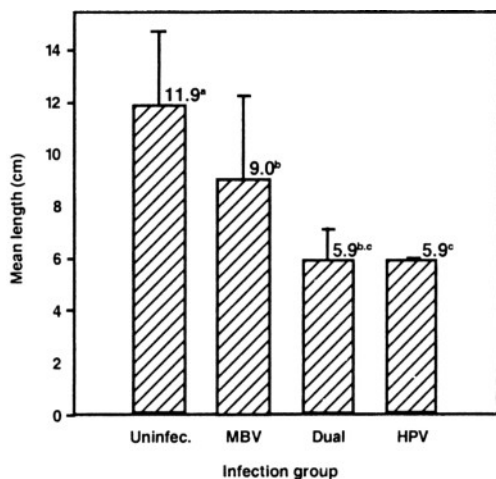
**Figure 2.** Scanning electron micrograph of MBV inclusion bodies purified from shrimp larvae by differential centrifugation of tissue homogenates. Such particles can be used for subsequent viral purification and nucleic acid extraction

**Figure 3.** Transmission electron micrographs of MBV. The upper left photograph shows two virions with intact envelopes on the left and right and one with a fractured envelope revealing the inner nucleocapsid. The lower right photograph shows a section of an inclusion body with rod shaped virions embedded in the polyhedrin matrix



Even though we knew that MBV was not a serious pathogen for the black tiger prawn, we still wanted to eliminate it from the farming system because we did not believe that the shrimp could carry such heavy viral infections without paying some price. Indeed, we have done further work showing that the mean length of MBV infected shrimp is significantly shorter than uninfected shrimp from the same pond (Fig. 4) (Flegel *et al.*, 2001), although this difference was not easily detected until late in the cultivation cycle. These results support findings from studies in the Philippines indicated that MBV infections could slow growth in intensive cultures. We also suspect that poor rearing conditions could lead to a flare-up of the virus followed by secondary bacterial infections resulting in shrimp death. In fact, it turned out the that virus could be eliminated from the rearing system by a combination of washing eggs and/or early naupliar stages with clean sea water, separate rearing of single-spawn larval batches and discard of occasionally infected batches of larvae or postlarvae (PL).

The experience with MBV had important spin-offs in terms of cooperation with other scientists working on shrimp diseases. It started our contact with Australian scientists through cooperation with Dr. Joan Vickers at the University of Queensland in what comprised our start on viral gene cloning work and DNA diagnostic probe development. It also started our interactions with Dr. S.N. Chen in Taiwan, Dr. D.V. Lightner in Arizona and Dr. J.A. Brock in Hawaii.



**Figure 4.** Comparison of infection status with length for uninfected shrimp and shrimp infected with MBV, HPV, or both MBV and HPV (dual). The shortest shrimp were those with HPV or dual MBV/HPV infections and the longest were those with no infection. Bars marked with different letters (a,b,c) indicate means that are significantly different ( $P < 0.05$ ) (Flegel *et al.*, 2001)

Two mixed primers for the detection of MBV by PCR amplification have been published (Chang *et al.*, 1993). These were designed based on conserved regions of the insect polyhedrin genes and they give rise to fragment of approximately 600 bp with MBV. These primers were also used to prepare the 600 bp fragment for use as a DNA probe for dot blot hybridization and for *in situ* hybridization. The primers are:

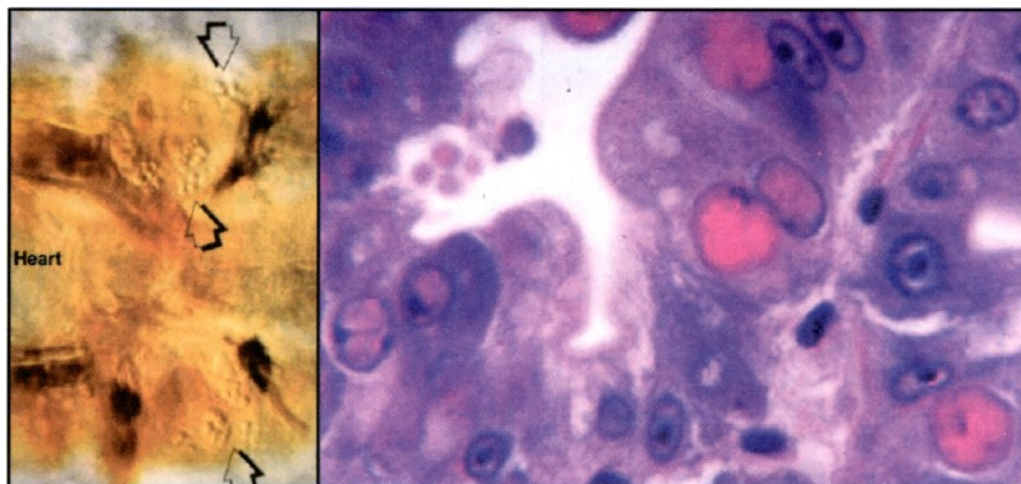
5'-AC(CT) TA(CT) GTG TAC GAC AAC AAA TA(CT) TAC AAA-3'  
 5'-GG(TC) GCG TGTG) GG(TC) GCA AA(CT) TC(CT) TT(TA) AC(TC) TT(GA) AA-3'

A better nested PCR process for MBV detection has been published from Australia (Belcher and Young, 1998). The primers were derived from a cloned fragment of DNA prepared from purified MBV. The two PCR products were 533 and 361 bp and sensitivity was very high at 0.01 fg MBV DNA in mixtures containing whole postlarval DNA. The primer sequences are:

1st round sense: 5' -CGA TTC CAT ATC GGC CGA ATA- 3'  
 1st round antisense: 5' -TTG GCA TGC ACT CCC TGA GAT- 3'  
 2nd round sense: 5' -TCC AAT CGC GTC TGC GAT ACT- 3'  
 2nd round antisense: 5' -CGC TAA TGG GGC ACA AGT CTC- 3'

Our work with MBV continues, albeit at a lower level of intensity. We have used the Belcher and Young (1998) probe for successful detection of MBV from shrimp feces by dot-blot DNA hybridization and PCR amplification. This would allow for non-destructive testing of broodstock and pond reared shrimp. More work is needed on the MBV genome particularly with respect to comparative analysis with the insect NPV for which much more information is currently available. Such studies should give some insight into the interaction between MBV and its shrimp host.

Since MBV is a DNA virus like WSSV and HPV (see below for details on these two viruses), it should be possible to devise a multiplex PCR method that would be capable of detecting any combination of these viruses in DNA extracts from PL. Since PL in Thailand are already regularly tested for WSSV by PCR, it would seem a worthwhile goal to assay for all of these viruses in a single PCR reaction.



**Additional figure.**

Photomicrograph on the left shows MBV occlusion bodies (arrows) as viewed directly through the cuticle of an early PL specimen using the light microscope with a 40x objective. The photomicrograph on the right shows early to late stages of MBV infection in an H&E stained tissue section of the hepatopancreas (HP). Nuclei become enlarged with an acidophilic (pink) center with nucleoli and chromatin condensed along the nuclear membrane. At the final stage the very enlarged nuclei contain acidophilic, paracrystalline protein inclusions (occlusion bodies). In this photomicrograph, 4 occlusion bodies can be seen free in the lumen of the HP where they have been discharged from a lysed cell

## YELLOW-HEAD VIRUS (YHV)

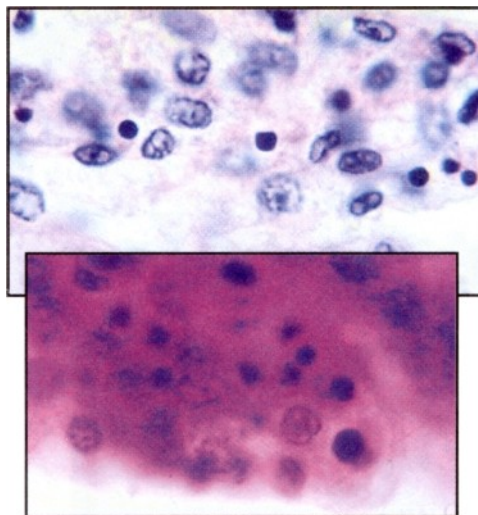
Our work on MBV was interrupted by the arrival of the first really serious viral pathogen of shrimp in Thailand in 1992. In retrospect, we know that this virus first began to cause problems in Thailand in 1990 (Limsuwan, 1991), but it was not discovered as a new pathogen until 1992 (Boonyaratpalin *et al.*, 1993; Chantanachookin *et al.*, 1993). The virus was named from the gross signs of disease which included a yellowish cephalothorax and very pale overall coloration of moribund, infected shrimp (Fig. 5). Histologically, it can be recognized by densely basophilic inclusions, particularly in the gills by rapid staining (Flegel *et al.*, 1997a) (Fig. 6) and the haemolymph (Nash *et al.*, 1995) (Fig. 7). Research on YHV in Thailand has been reviewed (Flegel *et al.*, 1997a; Flegel *et al.*, 1995b) along with current practices for diagnosis, prevention and control.

YHV was first thought to be a baculovirus but we discovered during purification and characterization that it had curious morphology (Figs. 8 and 9) and that it was an RNA virus (Wongteerasupaya *et al.*, 1995a). DNA diagnostic probes were prepared by cDNA preparation and cloning, although these currently work best in RT-PCR assays (Wongteerasupaya *et al.*, 1997) rather than *in situ* hybridization (Fig. 8), probably because of the instability of the viral RNA. Even with the PCR assay, samples must be processed quickly, since storage at  $-80^{\circ}\text{C}$  does not prevent deterioration of the RNA.



**Figure 5.** Gross signs of yellow-head infection are seen here in the 3 shrimp on the right. They are generally bleached in color with a yellowish discoloration of the cephalothorax ("head") region when compared to shrimp of normal appearance on the left

**Figure 6.** Gills of YHV infected shrimp stained with H&E in normal paraffin sections (upper plate) and in rapidly fixed and stained (3 hr) whole mounts (lower plate). The densely stained purple (basophilic) inclusions contrast sharply with the normal nuclei which are larger and show scattered staining of the chromatin. These gill inclusions are evident only in moribund shrimp and consequently do not have diagnostic predictive value



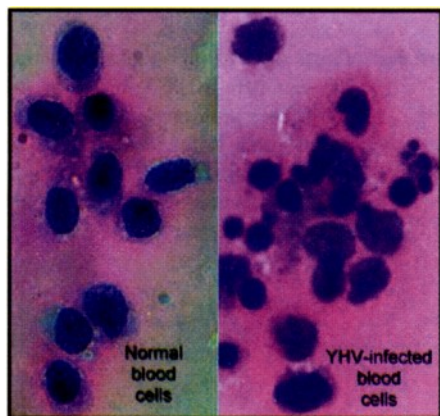
The PCR amplicon sequence and the primers for YHV detection in infected shrimp by RT-PCR are as follows (Wongteerasupaya *et al.*, 1997):

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CCG CTA ATT TCA AAA ACT ACG ACA GAA ACA CCG GCA TGT CCT GTT CTC TCA
CTG AAT TCC AGC TCT CTC TCT CTC ACA TCC TCT ACC GTT CTG AAG CAC AGC
GTA CTC CTG ACG ACT TCC TCG ACA TAA CAC CTT

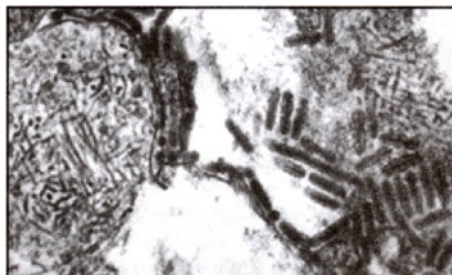
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**Figure 7.** Haemolymph from normal and YHV infected shrimp. The disintegration of the nuclei is clearly evident in the YHV infected shrimp. These can be seen in early stages of infection, but not later when the haemocyte population has been depleted by the virus. In addition, such disintegration can be found with some bacterial infections

**Figure 8.** Transmission electron micrograph of YHV-infected shrimp tissue showing the unusual filamentous nucleocapsid precursors (on the left) and mature, rod-shaped, enveloped virions (on the right)



**Figure 9.** Transmission electron micrograph of negatively stained purified virions of YHV. Note that the virus particles are enveloped and that the envelope has a halo of appendages characteristic of some RNA viruses

This fragment can also be labeled and used for dot blot or *in situ* hybridization assays. The problem with these techniques is that the viral RNA is very labile and must be protected during specimen preparation. For *in situ* hybridization using fixed tissues, it is important not to use Davidson's fixative which is acidic and will destroy the RNA. More successful *in situ* hybridization results using RNA-friendly fixation of shrimp tissues has been reported by Tang and Lightner (1999). Briefly this method uses a formula where the acetic acid in Davidson's fixative is replaced with additional formalin. After overnight fixation, the tissues (without cuticle, which cannot be cut unless decalcified) are quickly embedded in paraffin after which they can be stored indefinitely before *in situ* hybridization assays are carried out. However, once rehydrated, the specimen sections will be extremely vulnerable to attack by RNase and all precautions must be taken to protect the specimens. We have used neutral buffered formalin and Davidson's fixative with the acetic acid

replaced by distilled water as alternative fixatives to Davidson's and these appear to work equally well. It has also been proposed that normal Davidson's fixative may be used, so long as the fixation is not longer than overnight and followed immediately by dehydration and embedding in paraffin.

The Tang and Lightner (1999) probe used for *in situ* hybridization was a 1051 bp dioxygenin labeled probe derived from a 1061 bp YHV cDNA clone and produced from that clone template using PCR with the following primers:

Sense: 5' -ACA TCT GTC CAG AAG GCG TC -3'  
 Antisense 5' -GGG GGT GTA GAG GGA GAG AG -3'

They also gave primers derived from the same cDNA fragment for detection of a 273 bp YHV specific amplicon by RT-PCR:

Sense: 5' -CAA GAT CTC ACG GCA ACT CA-3'  
 Antisense: 5' -CGA CGA GAG TGT TAG GAG G-3'

In addition to these reagents, Cowley *et al.* (2000a) have published primer sequences that can be used for detection of both YHV and the related Australian lymphoid organ virus (LOV) (Spann *et al.*, 1995) and gill associated virus (GAV) (Spann *et al.*, 1998). These primers were designed from a 781 bp GAV cDNA clone to give a 618 bp RT-PCR product and are:

Sense: 5' -AAC TTT GCC ATC CTC GTC AC-3'  
 Antisense: 5' -TGG ATG TTG TGT GTT CTC AAC-3'

Together with Dr. Walker's group from CSIRO Australia, sequencing and comparison of the 618 bp RT-PCR fragments obtained using these primers with YHV, GAV and LOV has shown that all are closely related single stranded, positive sense RNA viruses that will likely be the first invertebrate representatives from the Order *Nidovirales* (Cowley *et al.*, 2000b). LOV and GAV shared approximately 95% DNA sequence homology and 100% amino acid homology establishing that they are the same virus strain, while GAV and YHV shared approximately 85% DNA sequence homology and 96% amino acid homology indicating that they are different strains (Cowley *et al.*, 1999). An excellent commercial kit is available from Intelligene of Taiwan that gives differential and graded RT-PCR detection for GAV and YHV.

Dr. Walker's group (CSIRO, Brisbane) has recently found a third, apparently non-pathogenic YHV variant in Viet Nam and Thailand that is closer in sequence to GAV than the original Thai YHV. Using the Intelligene kit, the new YHV strain gives the same PCR band as Australian GAV. These RT-PCR probes are also useful for examining suspected carries of YHV and testing whether they can transfer the virus to cultivated shrimp. The results of such studies will have an important impact on disease control programs for shrimp farmers. Dr. Walker's group has developed an excellent preservative solution for field samples. It contains 80% ethanol, 20% glycerol and 0.25% mercapto-ethanol (prepared by mixing 80 ml absolute ethanol, 20 ml pure glycerol and 0.25 ml of mercapto-ethanol). Samples are crushed or homogenized in this at 1 part sample to 10 parts preservative and survive storage for a reasonable length of time at room temperature. At the laboratory, supernatant preservative is removed before nuclei acid extraction (RNA or DNA) from tissues in the usual manner for use as a PCR template.

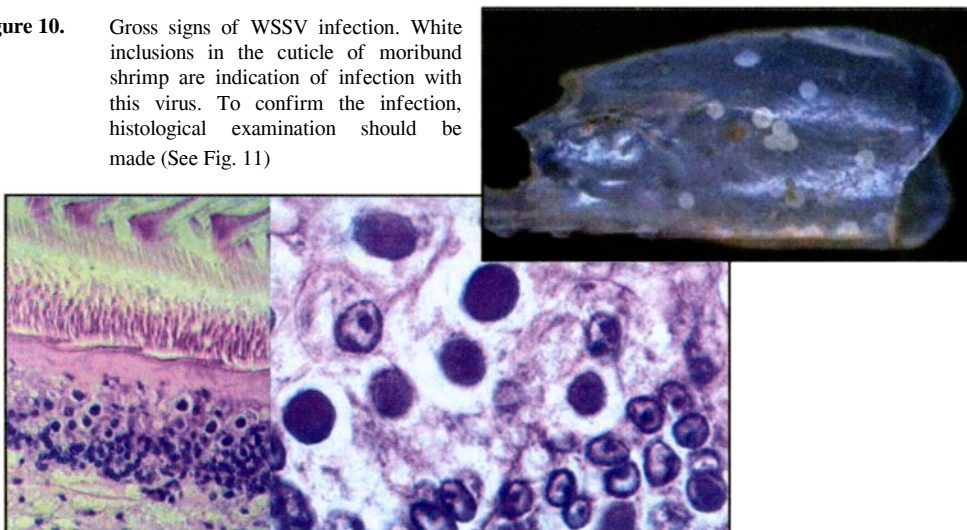


## WHITE-SPOT SYNDROME VIRUS (WSSV)

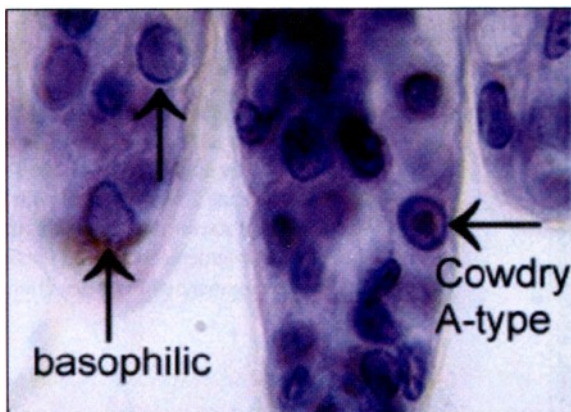
Historically, this was the second viral infection to seriously affect Thai shrimp farmers. We have recently reviewed the studies and control methods for this virus in Thailand (Flegel et al., 1997a). Infections with it usually give gross signs of white inclusions of various sizes embedded in the carapace at the late stages of infection (Fig. 10). These characteristic gross signs of infection were first reported from an outbreak which occurred in *P. japonicus* in Japan in 1993. The causative agent was a new bacilliform virus (Takahashi et al., 1994). In the same interval viral infections with similar gross signs were seen in *P. japonicus*, *P. monodon* and *P. penicillatus* in Taiwan and China (Chou et al., 1995). With hindsight, we now know that in Thailand the virus was first seen in laboratory reared *P. monodon* in late 1993 (Wongteerasupaya et al., 1995b). Now, the virus is called white spot syndrome virus (WSSV) by general consensus (Lightner, 1996; Lightner and Redman, 1998). However, it was not found in Thai farmed shrimp until late 1994, when mass mortalities began to be reported with characteristic gross signs of WSSV infection (Wongteerasupaya et al., 1996).

We originally called WSSV a baculovirus based on its cylindrical morphology and histological lesions that resembled those of "non-occluded" baculoviruses (Wongteerasupaya et al., 1995b) and in one publication, we were actually requested by the reviewers to call it PmNOBII for "*Penaeus monodon* non-occluded baculovirus II" (Wongteerasupaya et al., 1996). In the end, this turned out to be a mistake. We now know that WSSV is a tailed, rod-shaped, double stranded DNA virus with a very large circular genome in the order of 300 kpb that is available at GenBank (Lightner, 1996; van Hulten et al., 2001; Wongteerasupaya et al., 1995b). Since the genome has no significant homology to any known virus, a new viral family (*Nimaviridae*) and genus (*Whispovirus*) have been proposed for it (van Hulten et al., 2001).

**Figure 10.** Gross signs of WSSV infection. White inclusions in the cuticle of moribund shrimp are indication of infection with this virus. To confirm the infection, histological examination should be made (See Fig. 11)

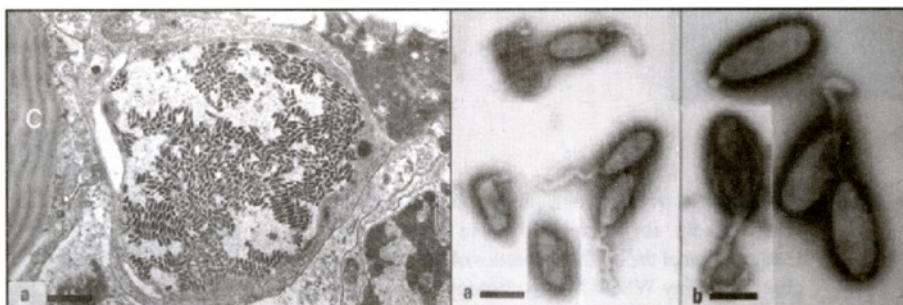


**Figure 11.** Histopathology of WSSV. The low magnification micrograph on the left shows many characteristic inclusion of WSSV under the cuticle of the gut epithelium. The high magnification on the right clearly shows the hypertrophied nuclei. Note the nucleus in the lower right corner with a densely red stained center (acidophilic) surrounded by a clear space and then a ring of purple (basophilic) chromatin. This is a Cowdry A-type inclusion characteristic of nuclei in the early stages of infection



**Figure 12.** Rapidly stained whole gill fragment showing typical WSSV histopathology. This technique can also be used with sub-cuticular epithelial tissue for rapid histological confirmation of WSSV infections. Note the Cowdry A-type inclusion in the nucleus on the far right. The other nuclei, at later stages of infection have more basophilic centers

On the basis of gross signs of disease, histopathology with the light (Figs. 11 and 12) and electron microscopes (Fig. 13), and DNA characteristics (Fig. 14), it soon became obvious that these infections could be ascribed to the same virus or closely related forms of it (Chou *et al.*, 1995; Durand *et al.*, 1996; Kimura *et al.*, 1996; Wang *et al.*, 1995; Wongteerasupaya *et al.*, 1995b). This contention was further supported by *in situ* DNA hybridization tests with white-spot syndrome, cultivated shrimp of various species from several Asian countries (Wongteerasupaya *et al.*, 1996) (Fig. 15).

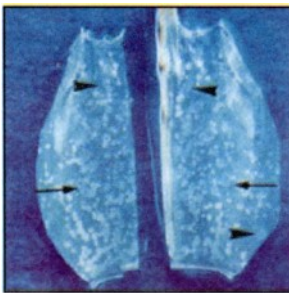


**Figure 13.** Transmission electron microscopy of WSSV. On the left is a low magnification view of a WSSV infected nucleus from gill tissue showing large numbers of rod shaped virions. On the right is a view of negatively stained enveloped virions showing unusual appendages and somewhat variable morphology



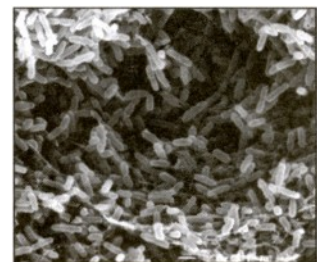
**Figure 14.** Polyacrylamide gel electrophoresis of nucleic acid from WSSV. Lane M contains a DNA marker and Lane 3 contains the undigested nucleic acid. The nucleic acid in Lane 1 was digested with BamHI while that in Lane 2 was digested with EcoRI. The EcoRI digest was cloned and screened for fragments that was specific for WSSV. The selected fragments were used for *in situ* hybridization assays and for development of PCR detection assays

It is extremely important to understand that diagnosis for WSSV infection cannot be based on the gross signs of white inclusions in the cuticle. A recent report by Wang *et al.* (2000) has shown that bacterial infections of the cuticle can also be associated with the formation of white inclusions, in the absence of WSSV infection (Fig. 15). Since the management response to bacterial and viral infections is fundamentally different, it is always necessary to confirm whether shrimp with such gross signs also show the histopathology characteristic of WSSV. This can be done by microscopic examination of whole gill fragments that have been rapidly fixed and stained in a simple, inexpensive process that takes only 3 hours.



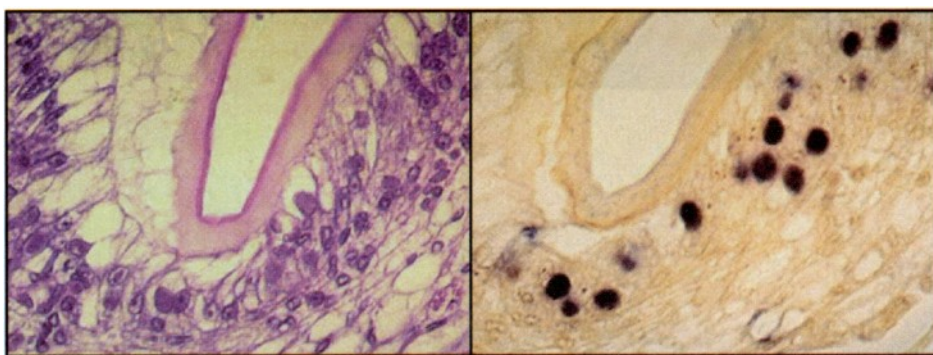
**Figure 15a.** Gross appearance of the carapace of shrimp showing bacterial white spot syndrome. These spots cannot be easily distinguished from those caused by WSSV, so histological examination is always required in the confirmation of WSSV infections (from Wang *et al.*, 2000)

**Figure 15b.** Scanning electron micrograph of bacteria colonizing the white spots in bacterial white spot syndrome. Examination of the epithelial tissues of the shrimp showed absence of any WSSV histopathology. The shrimp were also negative for WSSV by nested PCR assay (Wang *et al.*, 2000)

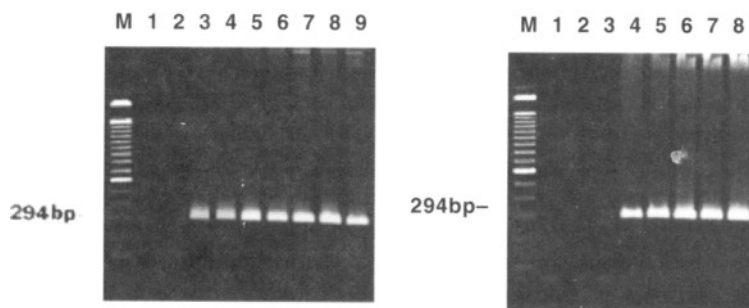


We developed DNA hybridization probes for WSSV (Wongteerasupaya *et al.*, 1996) (Fig. 16) and soon thereafter the primers for detection of WSSV by PCR, although the primer sequence was not published (Kanchanaphum *et al.*, 1998) but they are available commercially from the Shrimp Biotechnology Business Unit, National Center for Genetic Engineering and Biotechnology, Bangkok. These primers are widely used in Thailand for screening broodstock and PL in an attempt to stem the spread of the WSSV and restore production to former levels. The rapid implementation of this PCR screening system together with other appropriate management (Chanratchakool and Limsuwan, 1998; Flegel *et al.*, 1997a; Withyachumnamkul, 1999) has probably rescued the Thai shrimp industry from a disaster similar to that which occurred in China in 1993. By our reckoning, the preventative measures have probably saved the country in the order of 1 billion US\$ per year in export earnings since 1995. Again we are cooperating with Dr. Walker's group in Australia and with scientists in Taiwan and Japan in the analysis of viral DNA sequences from various sources, in order to understand the relationship amongst the epizootics that are occurring throughout Asia and now the Americas.

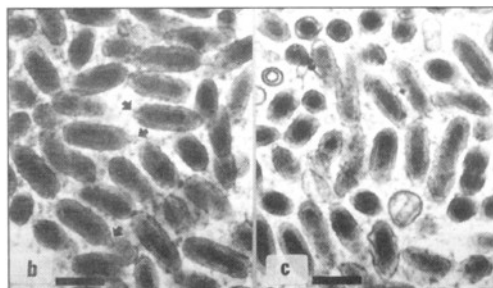
Another use of the PCR test has been to identify and monitor the transfer of WSSV from reservoir hosts to shrimp. In these studies, the most surprising feature has been the wide range of potential hosts. WSSV infects not only several species of penaeid shrimp including those cultivated in the western hemisphere (Lu *et al.*, 1997), but apparently also a wide range of other decapods, including crabs and more distantly related crustaceans such as copepods and perhaps even aquatic insect larvae (Lo *et al.*, 1996a; Lo *et al.*, 1996b). For shrimp farmers, it is extremely important to establish whether these non-cultivated crustaceans are *bona fide* reservoirs of the virus that can transmit it to cultivated shrimp. Studies in Thailand (Supamattaya *et al.*, 1998) have proven that the swimming crab, *Portunus pelagicus*, and the mud crab, *Scylla serrata*, can be infected with WSSV by injection or feeding, and we have shown by time-course PCR assay, histopathology, *in situ* DNA hybridization that *P. pelagicus*, and the mangrove crab can transmit the virus back to shrimp within a few days via water (Kanchanaphum *et al.*, 1998) (Fig. 17).



**Figure 16.** *In situ* hybridization detection of WSSV. The panel on the left shows H&E staining of WSSV infected tissue and that on the right shows a serial section of the same tissue assayed by *in situ* hybridization. The dark cells indicate positive hybridization



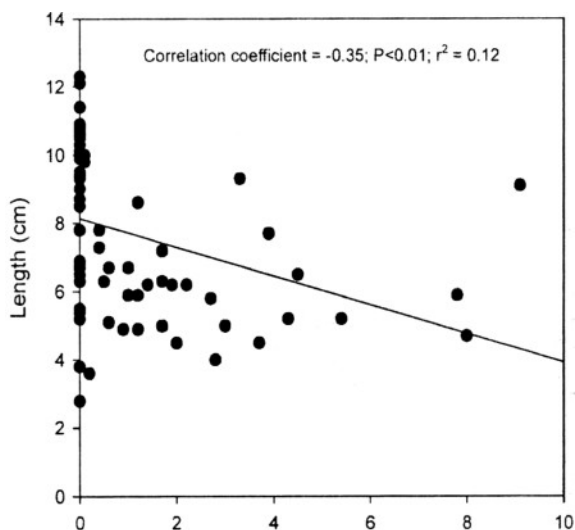
**Figure 17.** Examples of PCR amplification tests for WSSV in a reservoir host. Here on the left is seen an agarose gel with PCR products derived from samples of the crab *Sesarma* with a strong product band seen in lane 4 at 36 h after injection of the virus. The gel on the left shows the appearance of positive PCR bands for the presence of WSSV in the haemolymph of shrimp cohabitants with the infected crabs. The virus was transferred and became evident in the haemolymph by 24 h. Lane 1 = molecular marker; lanes 1-9 = products from haemolymph at 0 h (lane 1) and every 12 hours thereafter



**Figure 18.** Transmission electron micrograph of intranuclear WSSV virions at high magnification in a tissue section of an infected shrimp specimen

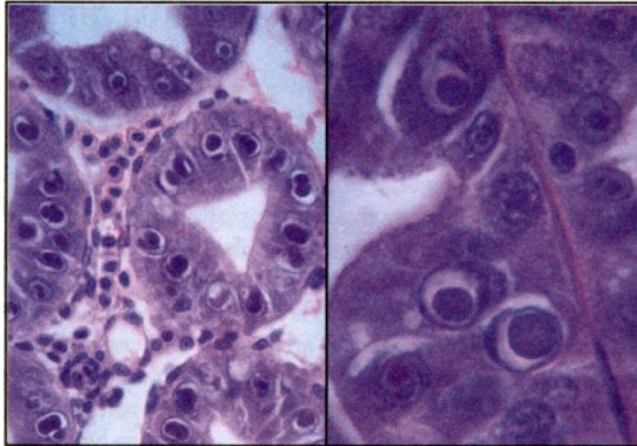
## HEPATOPANCREATIC PARVOVIRUS (HPV)

We have been interested in this virus for some years (Flegel *et al.*, 1992b; Flegel *et al.*, 1995a) but it has been hard to get information. Our preliminary data (Flegel *et al.*, 1995a) suggested to us that this virus was lethal to shrimp larvae during the interval of the first month after stocking. However, our most recent results (Flegel *et al.*, 1999) suggest that there is a strong statistical correlation between HPV infection and small size, suggesting that most HPV-infected shrimp simply grow very slowly and stop growing at around 6 cm in length (Figs. 4 and 19). Thus, when the shrimp are sampled by cast net, these non-growers escape and are usually counted as non-survivors. Functionally speaking for the farmer, this is almost equivalent to death since shrimp 6 cm in length weigh only about 5 g (200 pieces per kg) and have so little market value that they do not cover the cost of rearing. If HPV infected shrimp constitute a substantial part of a pond population, the resulting crop may be a financial loss. On the basis of our recent results, we are recommending that PL batches with moderate to high prevalence of HPV infections be rejected for stocking by shrimp farmers. However, there is no indication that HPV infections spread horizontally in growout ponds, so it may be acceptable to use PL batches with a low prevalence of HPV, if no uninfected batches are available and if the projected loss would be economically acceptable.

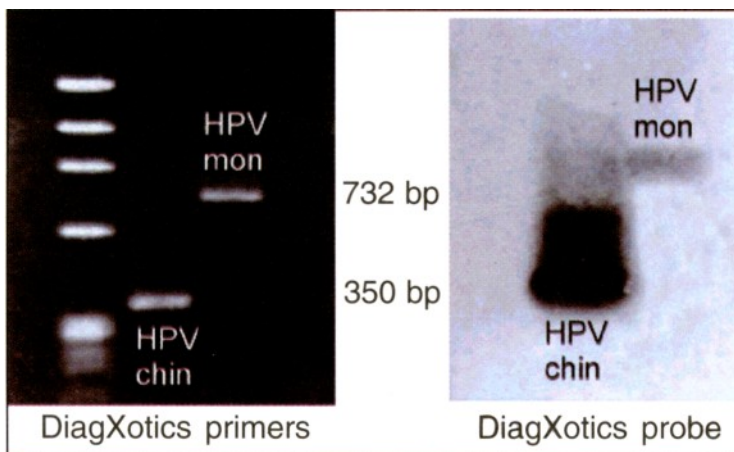


**Figure 19.** Severity of HPV infection versus length for shrimp from a farming system in Southern Thailand (Flegel *et al.*, 1999). Please also refer back to Fig. 4, where shrimp size is shown according to infection group. There, HPV infected shrimp comprise the groups with the smallest sizes as either a single or dual infection with MBV





**Figure 20.** Histopathology of HPV infections. The panel on the left shows a low magnification of hepatopancreatic tubule epithelial cells infected with HPV. It produces densely purple stained (basophilic) intranuclear inclusions. On the right, these inclusions are shown at high magnification together with crescent shaped nucleoli which they have pushed to one side of the nucleus



**Figure 21.** PCR assay of Thai HPV using primers from DiagXotics Co. Ltd. The gel on the left shows the control PCR product at 350 bp and the Thai product at 732 bp. The southern blot membrane on the right was prepared from the gel on the left and it shows that Thai HPV does not react so strongly with the DiagXotics labeled probe as does the DiagXotics control HPV product



Detection of HPV is not easy because there are no unique and distinctive gross signs associated with infection. Since it occurs only in the hepatopancreas (Fig. 20), the shrimp must be killed to be examined histologically. This is not problematic for larval samples, but it is for broodstock and pond reared shrimp. We need diagnostic reagents that can preferably be used with the shrimp feces, and DNA probes are ideal for such applications. Commercial DNA diagnostic probes and PCR primers for HPV are available (DiagXotics Co. Ltd., Wheaton, Conn.) based on an HPV isolate in *P. chinensis* (HPV-chin) from Korea, but it turned out that these were not ideal for HPV from Thailand. HPV in *P. monodon* (HPV-mon) from Thailand is quite different (Sukhumsirichart *et al.*, 1999) with a larger genome of 6 kb single stranded DNA compared to 4 kb of HPV-chin. The DiagXotics primer designed from HPV-chin DNA gives a 732 bp fragment with HPV-mon rather than the predicted one of 350 bp. When we compared the 732 bp fragment from HPV-mon to that of HPV-chin at GenBank, we discovered that HPV-mon had only 70% homology to HPV-chin (Phromjai *et al.*, 2001) and this explained the weak DNA hybridization in Southern blots (Figs. 21). To improve sensitivity with HPV-mon, the primers of Sukhumsirichart *et al.*, (1999) may be used instead of those of DiagXotics to yield an HPV specific fragment of 156 bp. This fragment is shown below with the primer sequences underlined.

```

5' - GCA CTT ATC ACT GTC TCT ACC CAA GTC ATG AGC TGT CTG
3' - CGT GAA TAG TGA CAG AGA TGG GTT CAG TAC TCG ACA GAC

AAA GCC TTG TAT ATA TGG CAA CCA GAC TTT GCT CAA GAA
TTT CGG AAC ATA TAT ACC GTT GGT CTG AAA CGA GTT CTT

ATC CTC CTT CAT GGT TAG CAT TTT CAC AGC TAT ACT AAT
TAG GAG GAA GTA CCA ATC GTA AAA GTG TCG ATA TGA TTA

CTT ATG ACA GAG CAA GGT ATT TAC AAA GTT CAC -3'
GAA TAC TGT CTC GTT CCA TAA ATG TTTCAA GTG -5'

```

One drawback of these primers is that they yield a very small PCR fragment that may be confused with primer-dimer pairs. As a result, we have designed a second set of primers for PCR amplification of a 441 bp specific DNA fragment from HPV-mon (submitted to DAO). These primers have also been used to produce a 441 bp dioxygenin labeled specific probe for HPV by PCR using the 732 bp cloned fragment as a template. These methods are currently being applied in Thailand for detection of HPV DNA in extracts derived from PL and faeces (Fig. 22) by PCR (Fig. 23) and dot blot hybridization (Fig. 24) and they are available commercially from the Shrimp Biotechnology Business Unit, National Center for Genetic Engineering and Biotechnology, Bangkok. The primer sequences are:

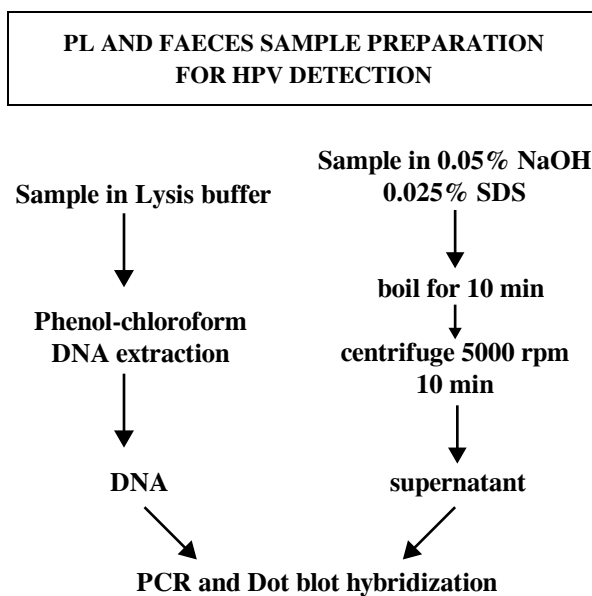
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primer HPV441F: 5'      ACA CTC AGC CTC TAC CTT GT 3'
primer HPV441R: 5'      GCA TTA CAA GAG CCA AGC AG 3'

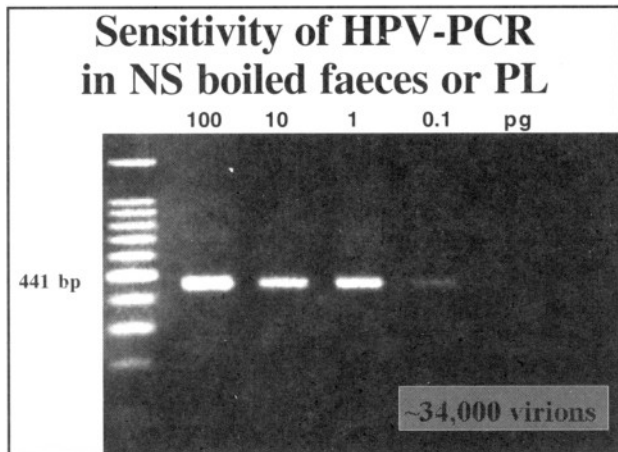
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and the PCR protocol is: 95°C for 5 min followed by 40 cycles of 95°C for 1 min, 60°C for 1 min and 72°C for 1 min with final extension at 72°C for 7 min. The expected amplicon is 441 bp. Other primer sequences and *in situ* probes have been described as well (Pantoja and Lightner, 2000, 2001).

One major mystery that remains unsolved is the source of HPV in the farming system. We have never seen it in hatchery larvae (unpublished) and there are, as yet, no published reports of it there. Nor did we find it in captive broodstock (Flegel *et al.*, 1997b) although the sample was small and we used histology only since we did not have PCR techniques at the time. The first place we have seen it with certainty is in nursery tanks to which the postlarvae are transferred from the hatchery for outdoor acclimatization before stocking in shrimp ponds. This suggests that there may be existence of an unknown reservoir carrier(s) of the virus. DNA probes would be useful in identifying this carrier(s) as a prelude to excluding it from the cultivation system.

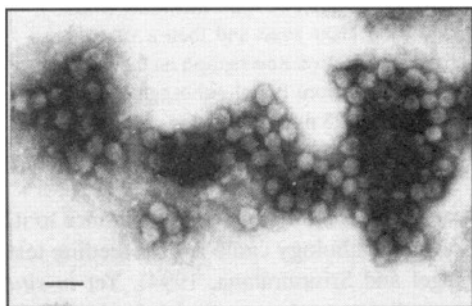
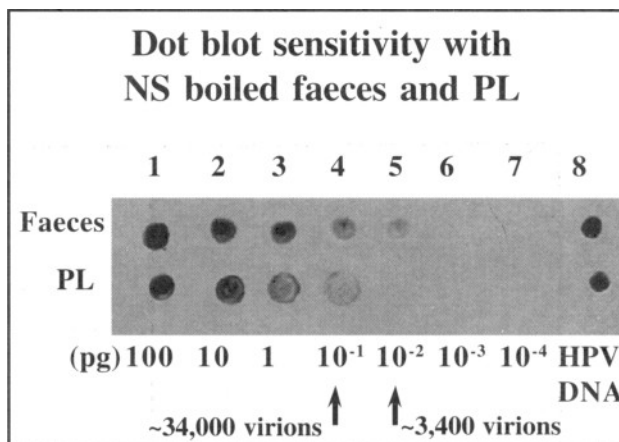


**Figure 22.** Procedures for preparation of DNA extracts from PL and faeces for PCR detection and dot blot detection of HPV and other DNA viruses. The lysis solution on the right can be used with fresh material only but has the advantage that no DNA extraction step is necessary. Normal lysis buffer (50 mM Tris-HCl pH 9, 100 mM EDTA, 50 mM NaCl, 2% SDS and Proteinase K 1 µg/ml added immediately before use) has the advantage that samples can be stored at room temperature for several years without degradation, but DNA extraction is necessary



**Figure 23.** Example of PCR detection results for boiled PL or faeces spiked with HPV DNA at various concentrations. Due to extraction losses and possible inhibitory substances, the sensitivity for was limited to approximately 34,000 virion equivalents per gram of original fresh weight sample

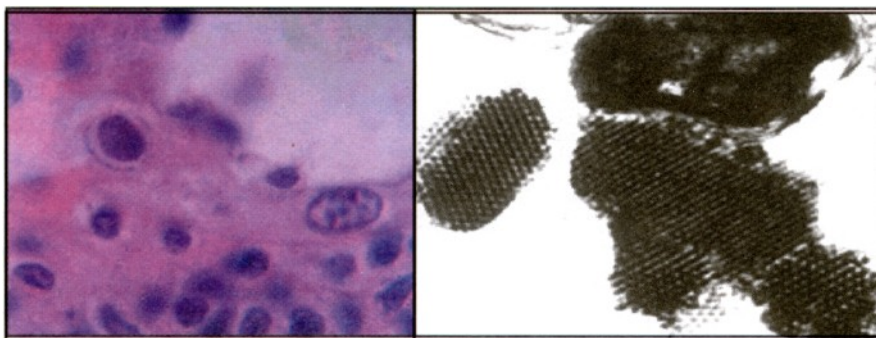
**Figure 24.** Example of a dot blot assay for HPV using fresh faeces or PL samples boiled in sodium hydroxide/SDS solution



**Figure 24a.** Negatively stained transmission electron micrograph of purified virus particles from HPV-mon. Bar = 50nm. Like other typical parvoviruses, the virions are unenveloped, icosahedral and very small (around 24 nm in diameter)

## INFECTIOUS HYPODERMAL AND HAEMATOPOEITIC NECROSIS VIRUS (IHHNV)

There is little hard data available for this virus in Thailand (Flegel *et al.*, 1995a). However there is some circumstantial evidence for its presence. First of all, IHHNV is probably endemic in Asian *P. monodon* (Lightner, 1996; Lightner and Redman, 1991). Secondly, we have found histopathology typical of the virus on one occasion in apparently healthy shrimp (Flegel *et al.*, 1992b) (Fig. 25) and the relevant specimens were confirmed for IHHNV by *in situ* hybridization (D.V. Lightner, unpublished). Third, we have frequently encountered paracrystalline arrays of 23 nm virus-like particles, which might be those of IHHNV, during examination of lymphoid organ specimens by electron microscopy (Flegel *et al.*, 1997b) (Fig 25). Fourth, Thai shrimp farmers have complained about the increasing tendency for wide size variation in harvested crops, a feature of IHHNV infection in *P. vannamei* in which IHHNV infection causes runt deformity syndrome rather than massive mortality (Lightner, 1993, 1996). We still do not know whether IHHNV has any impact on the Thai shrimp culture industry. The situation requires further study, and commercial DNA diagnostic probes developed for IHHNV in Dr. Lightner's laboratory are available for rapid detection by *in situ* hybridization or by PCR assay using shrimp haemolymph (DiagXotics, Wilton CT). We have tried these reagents and they do work with Thai material (Fig. 26), so there are no technical obstacles to the work.



**Figure 25.** IHHNV from Thai shrimp. The photomicrograph on the left shows one nucleus of the antennal gland with a typical IHHNV Cowdry-A type inclusion (red, acidophilic, central inclusion surrounded by a clear zone and then a ring of marginated purple, basophilic, chromatin). The electron micrograph on the right shows what may be IHHNV viral particle arrays from lymphoid organ tissue. The viral particles in the array are approximately 23 nm in diameter

One curious feature of this virus is what appears to be *P. monodon's* high tolerance to it. The Thai specimens in which we found typical IHHNV histopathology came from a feeding test group that exhibited normal growth and survival (Flegel and Sriurairatana, 1994). Yet *in situ* hybridization tests gave very strong positive reactions, indicating a heavy viral infection (D.V. Lightner, pers. com.). Unfortunately, no bioassay could be performed using *P. stylirostris* with these specimens. There are also anecdotal reports of IHHNV tolerant or "resistant" *P. stylirostris* from Tahiti which were found to transmit virulent IHHNV to naive *P. stylirostris* in bioassay trials

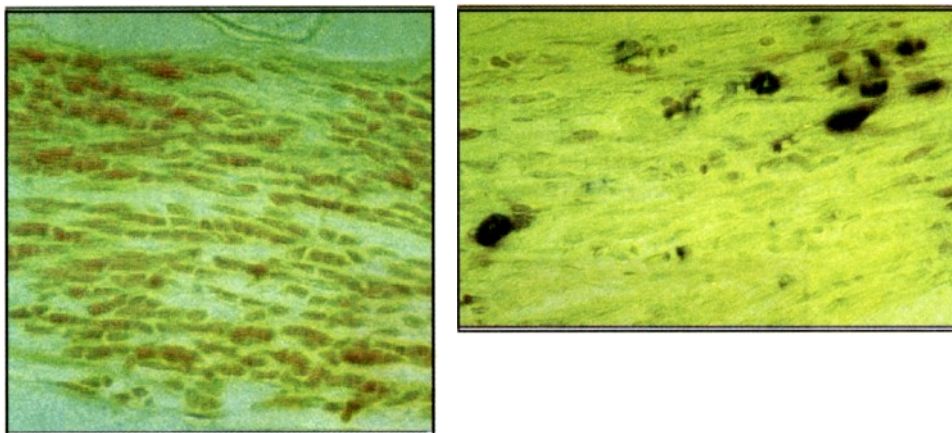
(D.V. Lightner, pers. com.). This scenario seems to have common features with the accommodation to WSSV and YHV in Thailand, and in the case of IHHNV, it is clear that the shrimp have changed, not the virus.

Primers for the PCR detection of IHHNV appear in the OIE Diagnostic Manual for Aquatic Animal Diseases (2000). These give a 356 bp IHHNV specific fragment. The sequence of the primers is as follows:

```
Sense:      5' - ATC GGT GCA CTA CTC GGA 3'
Antisense:  5'  TCG TAC TGG CTG TTC ATC 3'
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It is possible that PCR labeling of the 356 bp fragment would yield a probe suitable for either dot blot DNA hybridization or *in situ* hybridization, but this would have to be ascertained by appropriate testing.

The full sequence of IHHNV is now available at GenBank and we have done a comparison of our HPV-mon sequence and the full sequence of HPV-chin at GenBank with that of IHHNV. Although HPV and IHHNV are both parvoviruses, it was interesting to discover that there was no significant homology between the sequences of the HPV strains and that of IHHNV. We have also done comparative dot blots with HPV and IHHNV probes and targets and we have found no cross-hybridization.



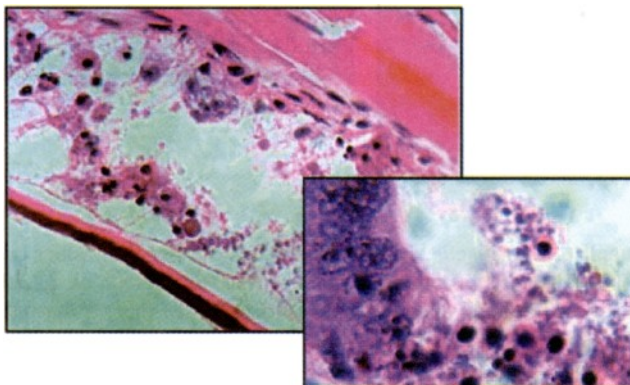
**Figure 26.** Example of a positive *in situ* hybridization reaction of DiagXotics commercial probe for IHHNV with shrimp from Thailand. The photomicrograph on the left shows a negative reaction with normal uninfected nerve tissue, while that on the right shows a positive reaction (blackened areas)

### TAURA SYNDROME VIRUS (TSV)

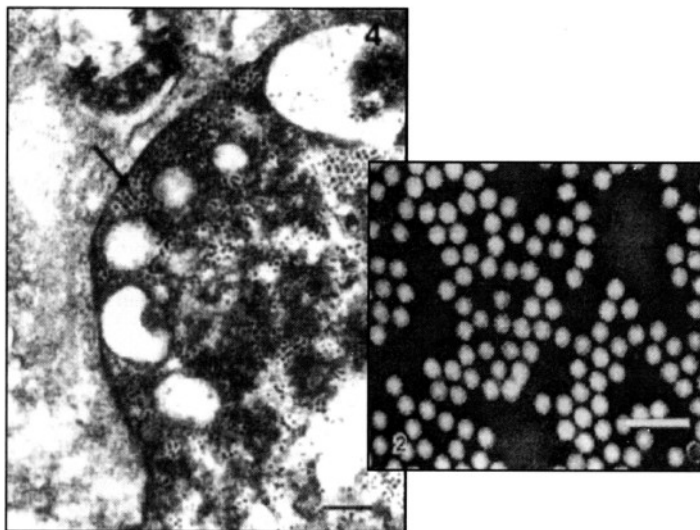
The newest viral pathogen to arrive on the Asian scene is Taura syndrome virus (TSV) (Lightner, 1996; Tu *et al.*, 1999). Taura syndrome was first recognized as a new disease in the Americas in 1992 but its viral etiology was not established until 1994 (Brock *et al.*, 1995; Brock *et al.*, 1997; Hasson *et al.*, 1995). The causative agent has been tentatively classified as a member of the Picorniviridae because it is an unenveloped, 32 nm icosahedral virus containing a 10.2 kb ssRNA genome of positive sense (Bonami *et al.*, 1997). TSV infections present characteristic gross pathology in *P. vannamei* that can serve from presumptive diagnosis (Fig. 27). These include reddening of the tail fan and visible necrosis of the epithelial tissue there in the acute phase of the disease. In the recovery or chronic phase of survivors, black lesions in the cuticle are often found. However, histological examination is required for confirmation of the disease (Fig. 28). By TEM icosahedral virions can be seen in the cytoplasm of infected cells (Fig. 29).



**Figure 27.** Gross signs of Taura syndrome. On the left is a tail fan of *P. vannamei* with reddish necrotic areas (arrow). The right photo shows black lesions in the cuticle characteristic of the chronic stage of TSV infection (Lightner, 1996)



**Figure 28.** Histopathology of TSV infected *P. vannamei* in the acute phase of infection. Note the large masses of spherical, cytoplasmic inclusions that begin as eosinophilic to light basophilic bodies and later become intensely basophilic



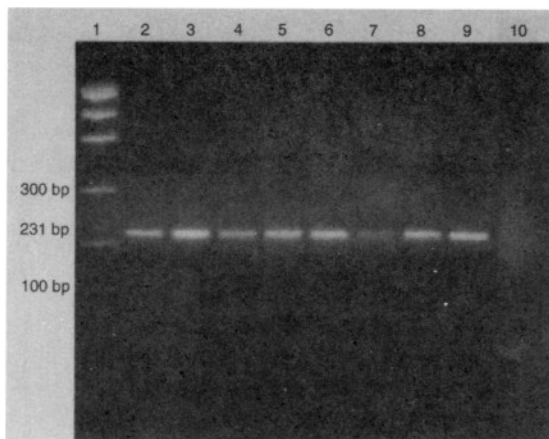
**Figure 29.** TSV by TEM. The electron micrograph on the left shows TSV in the cytoplasm by normal TEM using thin tissue sections from TSV infected *P. vannamei*. The micrograph on the right shows purified TSV virions by negative staining. (From Nunan *et al.*, 1998).

TSV outbreaks were first reported in Asia from Taiwan where *P. vannamei* had been imported live as fry and brooders for use in commercial aquaculture ponds (Tu *et al.*, 1999). It was probably introduced with the imported stocks but molecular epidemiological tests would be needed to confirm this. Although TSV is not highly lethal to *P. monodon*, its effect on other species of Asian shrimp is not known.

Molecular DNA methods for the detection of TSV have been reported (Mari *et al.*, 1998; Nunan *et al.*, 1998). Primers for an RT-PCR method that yields a 231 bp TSV specific fragment (Fig. 30) have been reported (Nunan *et al.*, 1998). The primers are:

Sense:           5' TCA-ATG-AGA-GCT-TGG-TCC     3'  
 Antisense:      5' AAG-TAG-ACA-GCC-GCG-CTT   3'

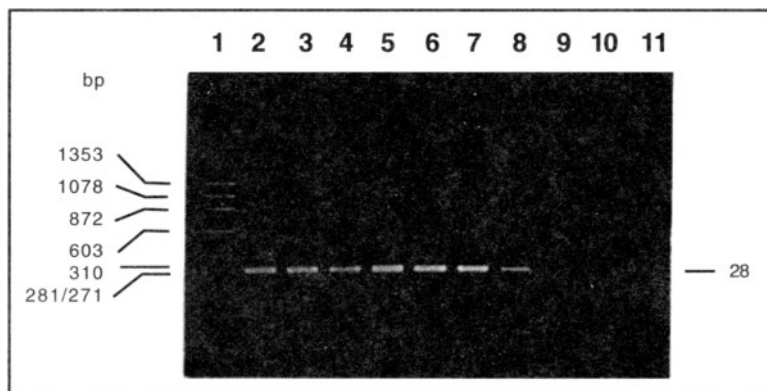




**Figure 30.** Agarose gel of PCR products from shrimp infected with TSV. Lane 1, 100 bp ladder; lanes 2-8, samples from shrimp injected with TSV and infection confirmed by histology; lane 9 positive TSV control; lane 10 negative control sample from pre-injection shrimp (Nunan *et al.*, 1998)

### ***VIBRIO PARAHAEMOLYTICUS* AND *V. PENAECIDA***

Species of *Vibrio* are often the cause of shrimp death, although we believed that they are usually opportunistic pathogens that overcome shrimp defenses when they are weakened by some sort of predisposing stress (Flegel *et al.* 1995a). The way to solve the problem is to remove the cause of the underlying stress. The main *Vibrio* species responsible for shrimp mortality are *V. parahaemolyticus*, *V. harveyi*, *V. vulnificus*, and perhaps *V. penaeicida*. We first worked on *V. parahaemolyticus* because it is also a serious human pathogen and a target for screening of frozen shrimp for export from Thailand. A probe was developed by random cloning and selection procedures and it was subsequently utilized for a rapid and sensitive PCR detection that can be used directly with shrimp haemolymph samples (Rojlorsakul *et al.*, 1998) (Fig. 31). This system can be used to non-destructively check shrimp haemolymph samples without the necessity of traditional steps for bacterial isolation, purification and nutritional testing to obtain an identification. Like the other PCR assays described, the process takes only a few hours and contrasts sharply with the traditional methods which require several days and may risk misidentification of atypical strains. It is hoped that a cheap multiplex system will eventually be developed which would allow for the simultaneous PCR assay for all of the major *Vibrio* pathogens, but this will probably take a few years to reach fruition.



**Figure 31.** Agarose gel of PCR products from shrimp haemolymph samples containing cells of *V. parahaemolyticus*. The highest sensitivity seen in the gel is for lane 8 using a sample containing  $2 \times 10^4$  bacterial cells per ml of haemolymph and 100 cells per PCR reaction vial

The PCR primers that yield the 285 bp fragment are as follows:

Sense: 5'-GTT ACG CAC AGA TGC GAC AT-3'  
 Antisense: 5'-CTT GTG GAT TGG ATT CTC GC-3'

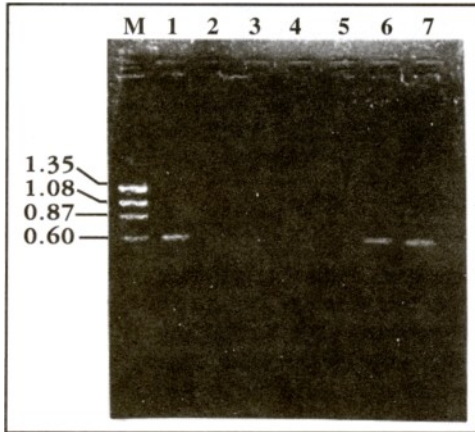
Another species of bacterium that appears to cause high mortality in *P. monodon* is *V. penaeicida* (Ishimaru *et al.*, 1995). This bacterium does not grow well on TCBS agar and may sometimes be overlooked in examining shrimp for bacterial infections. An RT-PCR method has been published for *V. penaeicida* from Japan (Genmoto *et al.*, 1996) and a PCR method has been published for *V. penaeicida* from New Caledonia (Saulnier *et al.*, 2000).

### THE MICROSPORIDIAN *AGMASOMA PENAEI*

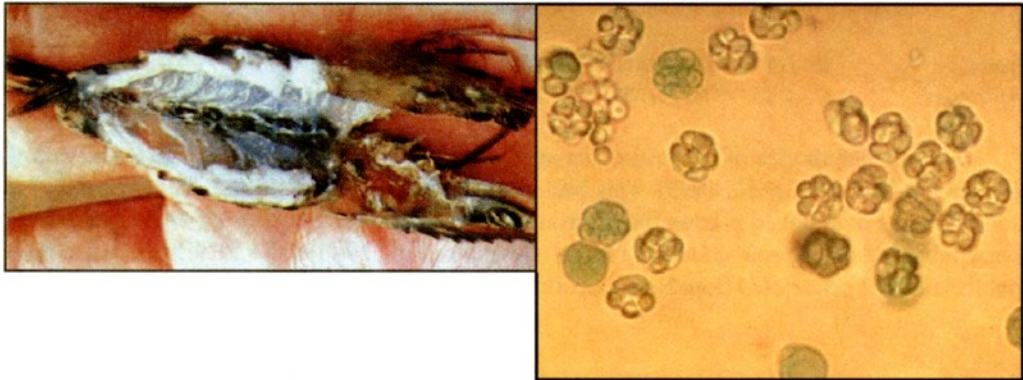
This intracellular parasite infects both *P. monodon* and *P. merguensis*, but apparently does not usually cause very high mortality (Flegel *et al.*, 1992a). It is most damaging because it disfigures the shrimp with white discoloration of the musculature ("white-back" or "cotton" shrimp) and reduces the selling price considerably. The parasite seems to be a bigger problem with *P. merguensis* and it is probably the major reason that it cannot be used as an alternate species to *P. monodon*, even when higher prices for it or its resistance to yellow-head virus infection, for example, would make its cultivation advantageous.

To address this problem, we have tried to find the source of the pathogen in the shrimp cultivation system. When direct infection tests between shrimp failed, we began to look for an intermediate host. To do this, DNA diagnostic reagents were developed and used to screen potential reservoir (*i.e.*, alternate) hosts (Pasharawipas and Flegel, 1994; Pasharawipas *et al.*, 1994; Pasharawipas *et al.*, 1997). By this process, two fish species (*Scatophagus argus* and *Priacanthus*

*tayenus*) were identified as potential hosts (Fig. 32). However, bioassay tests with one of these (*Scatophagus argus*) and shrimp have not yet been successful in closing the life cycle of the parasite, so the issue of the alternate host is still open.



**Figure 32.** Agarose gel of PCR products using *Agmasoma*-specific primers with DNA extracts from *Scatophagus argus* and *Priacanthus tayenus* as the template. Lane M = molecular marker; lane 1 = 600 bp product from *Agmasoma* positive control template; lanes 2-5, no product from various negative controls including bacterial, protozoan and normal shrimp DNA templates; lanes 6 and 7, positive PCR product from the respective fish DNA templates



**Additional figure.** The photograph on the left shows the gross appearance of white shrimp tissue infected with the microsporidian *Agmasoma penaei*. The photomicrograph on the right shows a fresh mount from infected tissue with immature sporoblasts stained with malachite green while mature sporoblasts with 8 spores each are refractory to staining

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