PATHOGENESIS OF GILL ASSOCIATED VIRUS DISEASE
ON PACIFIC WHITE SHRIMP – P. vannamei

by

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Master’s dissertation submitted in partial fulfillment of the requirements
For the degree of Master of science in Aquaculture
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Ghent, 24th August 2012

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ABSTRACT

The course of gill-associated virus (GAV) infections in *P. vannamei* has not been studied in detail up to today. The aim of the present study was to follow the pathogenesis of a well-characterized GAV stock in Specific pathogen free (SPF) Pacific white shrimp *Penaeus (Lipopenaeus) vannamei* under standardized conditions. Subadults (10-20 g n=20) were challenged by intramuscular injection with 50 µl/shrimp of 10^3 SID50 of (GAV). During two weeks of time course experiment, neither mortality nor typical gross signs of yellow head disease (YHD) were observed. Abnormalities in GAV infected shrimp were anorexia, soft shell and pale color in just 10% of animals. By IHC, GAV infection in *P. vannamei* was systemic with positive signals occurring in the cytoplasm of cells in most major organs. The lymphoid organ was the primary target organ of GAV infection, with the earliest visibly positive signals at 24 hours post injection (hpi). Histopathology showed that lymphoid organ spheroids (LOS) formed from 72 hpi and remained until the end of the experiment at 336 hpi. At the later phase of infection (from 120 hpi onwards), signs of apoptosis or necrosis with pyknotic and karyorrhectic nuclei and vacuolated cells were commonly observed in the LOS, but infrequently in gills and interstitial connective tissue of hepatopancreas. GAV infections in the lymphoid organ cells of infected shrimp during the time course were quantified as percentage of positive (+) signal per area. GAV replicated slowly at the early phase of infection (from 0 to 48 hpi) but rapidly increased from 72 hpi with 17% infected area. The intensity of GAV infection reached the highest of 30% positive signal area at 120 hpi. Later, the infected areas were gradually reduced to about 27%, 25% and 22% at the time points of 168, 240 and 336 hpi, respectively. Overall, GAV infection in subadult *P. vannamei* appeared to progress subclinically but persisted chronically, mainly in lymphoid organ. Some of our histopathological and IHC data were conflicting with reports from other scientists. Together with some peculiar infection patterns were observed in this study, these findings warrant further investigations.
CHAPTER 1 - INTRODUCTION

The Pacific white shrimp *Penaeus (Lipopenaeus) vannamei* has overcome the giant black tiger shrimp *P. monodon* to become the leading shrimp species in worldwide production contributing more than 50% of global farmed-shrimp production. (FAO, 2010) For example, in 2007, *P. vannamei* production accounted for more than 75% of global production (Wyban, 2009). Currently, *P. vannamei* has been the most cultured shrimp species in Asia and accounted for more than 95% of the total production in the Americas (Lightner, 2011). However, the global shrimp culture has faced with serious economic losses annually caused by disease outbreaks. Among of causative agents, virus is regarded as the most threatened accounted for 60% losses (Flegel, 2012). To date, about 20 viruses have been reported as shrimp pathogens, and six are listed by the World organization for animal health (= Office International des Epizooties or the OIE) due to causing notifiable diseases and devastating losses. The major viruses of concern are white spot syndrome virus (WSSV), Yellow head virus (YHV), Taura syndrome virus (TSV), Infectious hypodermal and haematopoietic necrosis virus (IHHNV), Infectious myonecrosis virus (IMNV), Gill associated virus (GAV), Monodon slow growth virus (MSGS) and Monodon baculovirus (MBV) (Walker et al., 2009).

**Yellow head complex virus** (YHCV) - the causative agent of yellow head disease (YHD) - is invertebrate nidovirus that is highly pathogenic for marine shrimp. From the first outbreak reported in Thailand in 1990, YHD has since been reported in most major shrimp farming countries in Asia, including India, Indonesia, Malaysia, Philippines, Sri Lanka, Vietnam and Taiwan (Flegel 2012). YHCV is an enveloped, rod-shaped (+) ssRNA; to date, six genotypes of the virus were notified and classified into the new genus Okavirus, the new family Roniviridae, belonging to the order Nidovirales (Walker et al., 2005). Among them, yellow head virus - YHV (YHV-type 1) and gill associated virus - GAV (YHV-type 2) are considered as virulent pathogens of penaeid shrimp. The other four known genotypes in the complex have been detected only in healthy *P. monodon* in Asia and not known to be associated with disease.

**Gill associated virus** (GAV) or YHV-type 2 is named following the disease outbreak in Australia in 1995-1996 causing mortalities and losses in farmed *P. monodon*. Currently, GAV has been also founded in healthy shrimp with very high prevalence (reach 100% according areas) and deemed to be related to mid-crop mortality syndrome (MCMS)
outbreaks and decreased production of shrimp culture in Australia (Oanh et al., 2011; Munro et al., 2010).

Although, yellow head disease outbreaks remain a serious concern for shrimp farmers, these pathogens are poorly understood with relatively little information yet available on either the epidemiology of YHD or the molecular biology of YHCV infection on penaeids (Sittidilokratna et al., 2006). Moreover, a little information is available on YHCV infection on *P. vannamei*, the most important and worldwide cultured species. Due to poorly comprehensive information, until now, there have been still no effective strategies to prevent and control YHD outbreaks. Although GAV is claimed to be far less virulent compared with the relative YHV-type 1 (Wijegoonawardane et al., 2008; Flegel, 2009), it still causes mortalities and losses for shrimp culture. Thus, it is essential to do research to give deeper and trustworthy information of YHCV in general and GAV infection on *P. vannamei* in particular.

To accomplish the Master program, a study of “Pathogenesis of gill associated virus disease on Pacific white shrimp *P. vannamei*” was conducted at Department of Virology, Faculty of Veterinary Medicine at Ghent University, from 3/2012-8/2012.

In the study, the cause, development, and effects of a disease of GAV on *P. vannamei* are investigated. Detailed understanding of GAV infection may lead to the development of effective disease control strategies.

**General objective**

Study on pathogenesis of gill associated virus disease on Pacific white shrimp *P. vannamei* - *P. vannamei*

**Specific objectives**

- Detect clinical signs and target organs of GAV infected shrimp
- Determine histopathological features of target tissues and organs in GAV infected shrimp
- Investigate histopathological progresses of GAV disease in the lymphoid organ of infected shrimp
- Virus quantification in the lymphoid organ of GAV infected shrimp
CHAPTER 2 - LITERATURE REVIEW

2.1. Aquaculture and shrimp culture industry

Global fish and shellfish aquaculture production has grown substantially, from 32 million tonnes in 2000 to 60 million tonnes in 2010. And for 2011, the production has continued to increase to 63.6 million tonnes (FAO, 2012). The Global Aquaculture Alliance has predicted that the average annual growth rates will continue to increase in 2012 and 2013.

Crustacean culture is an important part of the global fishery market due to the high value. For instance, in 2008, crustacean culture occupied only 9.5% of global aquaculture yield but accounted for 23.1% of total value (22.7 billion US$) (FAO, 2010). Among cultured crustacean species, shrimp is considered as the largest seafood commodity by value, accounting for 17% of total traded fishery products (Walker et al, 2010). The FAO and a GOAL survey estimate that the global production of farm-raised shrimp will reach 3.8 million tonnes in 2012 and 4 million tonnes in 2013 (Valderrama et al, 2012).

![Figure 1. Cultured shrimp production by world region from 1991 - 2013](image)

The Pacific white shrimp *Penaeus (Litopenaeus) vannamei* has been one of the most important and popular species in aquaculture due to fast growth, low production cost, potentiality for intensive culture and resistance to diseases. For instance, in 2008, the global production of *P. vannamei* accounted for 35% of all production coming from aquaculture (FAO 2010). The species has been mainly cultured in America since 1969 but after 2000, it
was introduced in Asia. By 2004, *P. vannamei* had overcome the Giant black tiger shrimp *P. monodon* to become the leading shrimp species in worldwide production contributing more than 50% of global farmed-shrimp production (reached 71% in 2011 according to FAO). From 2007, *P. vannamei* production accounted for more than 75% of Asian shrimp production and was the dominant species farmed in Thailand, China and Indonesia – the world’s three leading producing countries (Wyban 2009). Currently, in the Americas, *P. vannamei* accounts for more than 95% of the total shrimp production. (Lightner 2011)

![Figure 2. World shrimp culture (including *M. rosenbergii*) by species from 1991 - 2013](image)

**2.2. *Penaeus vannamei***

**2.2.1. Taxonomy**

The Pacific white-legged shrimp *P. vannamei* belongs to the Phylum Crustacea, in the Order Decapoda (Hickman et al, 2004).

<table>
<thead>
<tr>
<th>Kingdom</th>
<th>Animalia, Linnaeus 1758</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phylum</td>
<td>Arthropoda, Latreille 1829</td>
</tr>
<tr>
<td>Subphylum</td>
<td>Crustacea, Brünnich 1772</td>
</tr>
<tr>
<td>Class</td>
<td>Malacostraca, Latreille 1802</td>
</tr>
<tr>
<td>Subclass</td>
<td>Eumalacostraca, Gröbben 1892</td>
</tr>
<tr>
<td>Superorder</td>
<td>Eucarida, Calman 1904</td>
</tr>
<tr>
<td>Order</td>
<td>Decapoda, Latreille 1802</td>
</tr>
<tr>
<td>Suborder</td>
<td>Dendrobranchiata, Bate 1888</td>
</tr>
</tbody>
</table>
2.2.2. Morphology

The Pacific white shrimp can reach a maximum total length of 230 mm.

Shrimp body is divided into two parts, the cephalothorax and the abdomen. The cephalothorax is a result of the fusion of the head with the thorax. The cephalothorax is protected by a shell called the carapace, and consists of 13 sections. These sections each have appendages, including the rostrum, a pair of compound eyes, a mouth with strong jaws (mandibles), a pair of antennae and antennule, maxillae, maxillipeds, and 5 pairs of walking legs (pereiopods).

The abdomen consists of 6 abdominal segments, of which the first five bear a pair of swimming legs or swimmerets (pleopods). The sixth segment forms a tail fan (uropods) with a sharp spike in the middle (telson).

Most organs are located in the cephalothorax, such as gills, heart, hepatopancreas, antennal gland, lymphoid organs and stomach. Only the gut and reproductive organs are in the abdomen.

![Figure 3. Lateral view of the external anatomy of Penaeids (Bailey- Brock and Moss, 1992)](image-url)
2.2.3. Habitat and biology

The shrimp *P. vannamei* is a native American species distributed into the Eastern Pacific coast from Sonora, Mexico in the North, through Central and South America as far South as Tumbes in Peru, where the water temperature is almost warmer than 20°C all over the year.
Adult shrimp live and spawn offshore (up to 72 m depth) while larvae, migrate inshore to spend their stages of juveniles, adolescent and sub-adult near protected coastal areas such as estuaries, coastal lagoons and mangroves which serve as nursing areas rich in food sources (Bailey-Brock & Moss, 1992).

Penaeids become mature at the age of 6-7 months when the males get the body weight at 20g and the females reach a little bit bigger 28g onwards. Mating and spawning typically occur during the night. The maximum number of eggs at a spawning is around 1 million per female. A female weighing 30–45 g will spawn 100,000 – 250,000 eggs of approximately 0.22 mm in diameter.

Hatching occurs about 16 hours after spawning and fertilization. The first stage larvae, nauplii, are intermittent swimmer and phototaxis. Nauplii do not feed, but live on their yolk reserves. The next larval stages (protozoa, mysis and early postlarvae respectively) are still planktonic, phytoplanktonic and zooplanktonic consumers, and carried towards the shore by tidal currents. The postlarvae (PL) change their planktonic habit about 5 days after molting into PL, actively move inshore and begin feeding on benthic detritus, worms, bivalves and crustaceans. In about 4 months, the juvenile will develop into sub-adult stage which starts at the onset of sexual maturity when the shrimp migrate from inshore to offshore - the spawning grounds. After next 4 months, the adult stage is reachable, which is characterized by the completion of sexual maturity. (Briggs et al, 2004)

Figure 6. The life cycle of Penaeids. Eggs hatch within 16h after fertilization. The larval stages comprises nauplius (6 stages/2days), protozoa (3 stages/5 days), Mysis (3 stages/4-5days) and postlarvae (6-35 days). Transform from juvenile to sub-adult takes 135-255 days. The completed sexual maturity occurs within 10 months (Bailey-Brock et al, 1992)
2.3. Current major viral diseases and their impacts on shrimp culture

2.3.1. Major viral diseases in shrimp culture

More than 20 viruses have been reported as shrimp pathogens, and six viruses of marine cultured shrimp are currently listed as notifiable by the World organization for animal health (OIE) (OIE-2012) (Walker and Winton 2010, Stentiford, et al. 2012). The major viruses of concern are white spot syndrome virus (WSSV), yellow head virus (YHV), taura syndrome virus (TSV), infectious hypodermal and haematopoietic necrosis virus (IHHNV), infectious myonecrosis virus (IMNV), gill-associated virus (GAV), monodon slow growth virus (MSGS) and monodon baculovirus (MBV) (Walker and Mohan 2009).

Figure 7. History of the emergence of the major pathogens of farmed shrimp (Walker et al, 2009)

The emergence and spread of shrimp diseases are natural and predictable consequences of the nature of shrimp aquaculture activities (Walker, et al. 2009). There is no denying of the significant contribution of shrimp aquaculture to socio-economic development in developing countries. However, the rapid expansion of global shrimp farming has led to considerable environmental disturbances, changes in the ecology of pathogens, the use of artificial feeds with potential risks of cross species transmission of pathogens, culture intensification, exposure to stress etc. All this factors resulted in an increased pathogenicity of existing infections, exposure to new pathogens and the rapid transmission and transboundary spread of diseases (Lightner, 1996) (Walker, et al. 2009, Walker, et al. 2010). Among major viral shrimp pathogens, WSSV, TSV and IMNV have certainly emerged via cross species transmission (Walker, et al. 2009).
In the first quarter of 2012, the emergence of a new disease - termed “early mortality syndrome” (EMS) was pronounced in both *P. monodon* and *P. vannamei* cultured in Asia (Lightner, et al 2012 b). The disease was detected in shrimp farms located in southern China, Hainan Island, Vietnam and Malaysia. The disease appears within 20 to 30 days of stocking ponds with postlarvae. Mortalities can approach 100% in severely affected ponds, where diseased shrimp become lethargic and anorexic with the hepatopancreas organ appeared atrophied and necrotic. The cause of EMS is under investigation.

2.3.2. Socio-economic impacts of shrimp viral diseases

Infectious disease outbreaks have had devastating impacts on the shrimp farming industry due to lost crops, jobs and export revenue (Walker, et al. 2009, Senapin, et al. 2010, Walker, et al. 2010, Lightner 2011). Up to 2005, it was calculated that global production losses, caused by diseases over the preceding 15 years, had amounted to approximately US$15 billion, with about 80% occurring in Asia. The annual losses related to diseases in shrimp culture have been estimated to be about US$3 billion (Walker, et al. 2009).

Among infectious diseases, those caused by viruses stand out as the biggest causes for concern. Due to the incurability of the diseases and the far more problematic management than bacterial disease, up to 60% of crops are lost (Flegel et al, 2008) (Flegel 2012). For instance, during the first 10 years after its emergence in 1992, WSSV caused an estimated US$6 billion of economic losses in Asia alone (Lightner 1999). In the Americas, only in 1999, the disease outbreak of WSSV cost about US$ 2 billion. The outbreaks of YHV in Thailand in 1991-1992 caused a loss of US$500 million. At the same time, the emergence of TSV cost about US$3 billion for both Asia and Americas (Lightner 2011, Lightner, et al. 2012)(Walker, et al. 2010). The outbreaks of IMNV occurred in Americas and Asia in 2004 and 2006, respectively, were estimated the losses of more than US$1 billion (Lightner 2011, Lightner, et al. 2012). In 2010, aquaculture in China suffered production losses of 1.7 million tonnes caused by diseases, natural disasters and pollution. Disease outbreaks almost wiped out marine shrimp farming production in Mozambique in 2011 (FAO 2012).
Table 1. Estimated losses due to certain OIE-listed virus diseases since their emergence and/or discovery (Lightner, et al. 2012)

<table>
<thead>
<tr>
<th>Virus - region</th>
<th>Year of emergence</th>
<th>Product loss to industry</th>
</tr>
</thead>
<tbody>
<tr>
<td>IHNV – Americas*</td>
<td>1981</td>
<td>$0.5–1 billion</td>
</tr>
<tr>
<td>YHV – Asia</td>
<td>1991</td>
<td>$0.5 billion</td>
</tr>
<tr>
<td>TSV – Americas</td>
<td>1991/92</td>
<td>$1–2 billion</td>
</tr>
<tr>
<td>TSV – Asia</td>
<td>1999</td>
<td>$0.5–1 billion</td>
</tr>
<tr>
<td>WSSV – Asia</td>
<td>1992/93</td>
<td>$6 billion</td>
</tr>
<tr>
<td>WSSV – Americas</td>
<td>1999</td>
<td>$1–2 billion</td>
</tr>
<tr>
<td>IMNV – Americas</td>
<td>2004</td>
<td>$100–200 million</td>
</tr>
<tr>
<td>IMNV – Asia</td>
<td>2006</td>
<td>$1 billion (estimated)</td>
</tr>
</tbody>
</table>

* Includes the Gulf of California fishery for *Penaeus stylirostris* from 1990 to 1994 (Lightner, 1996b).

The most disastrous effects were often observed at the moment when a new major pathogen emerged, with a remarkable pause in the industry expansion or a dramatic drop in the level of production. For example, in China, after the emergence of WSSV in 1993, the production dropped down from 150,000 tonnes in 1992 to under 50,000 tonnes in 1994, costing about US$420 million. Also WSSV disease outbreaks caused a significant fall in the shrimp production in Ecuador, from 135,000 tonnes in 1998 to 50,000 tonnes in 2001 (Walker, et al. 2009).

Beyond the obvious direct negative effects on production and profitability, disease outbreaks also caused impacts on the income, food security, job security of farmers, especially for small scale farming. As a consequence, the sustainability of local communities and national economics were inevitably impacted and long term profitability remains under threat. (Walker, et al. 2010)

2.3.3. Environmental impacts

It is complicated to assess the total impact of aquatic animal diseases on the environment. However, two of the indirect environmental impacts of shrimp diseases are obviously evident. First, successive crop failures have led to the abandonment of cultured areas and the relocation of farming to new areas, which is causing big problems for coastal zones, especially mangrove habitat degradation. Second, the use of antibiotics, disinfectants and other chemicals to prevent, treat and control disease outbreaks have resulted in public concerns for pathogen resistance and pollution.
2.4. Yellow head disease and the yellow head virus complex

2.4.1. Historic emergence and distribution of YHV, GAV and other related viruses

Yellow head disease (YHD) was first described in 1991 in farmed *P. monodon* in central Thailand and subsequent disease outbreaks were reported from other shrimp farming countries in Asia, such as India, Indonesia, Malaysia, Philippines, Sri Lanka, Vietnam and Taiwan (OIE, 2009). The total losses of Asian shrimp industries, directly and indirectly - was estimated to be about US$500 million (Lightner 2011). The causative agent was found and named ‘yellow head virus’ (YHV) due to the remarkably typical gross sign of yellow/brownish discoloration of the cephalothorax and gills. The virus attacked early juvenile to sub adult shrimps and was soon considered as one of the most virulent shrimp viruses, causing total mortalities within 3-5 days of first appearance of visible signs of the disease. This original, highly virulent strain of YHV is now referred to as YHV type 1.

In 1995 - 1996, a virus resembling YHV was first reported in a disease outbreak of Australian farmed juvenile and sub adult *P. monodon* (Spann, Cowley, Walker and Lester 1997a). The clinical signs of the disease included a reddened colour of the body and gills, and mortality rose to 100%. Although these signs were not the same as those previously reported for YHD in Thailand, the histological lesions and morphological virions were indistinguishable between the new causative agent and YHV. An extensive degeneration and cellular necrosis were observed in the lymphoid organ and gills, in which YHV-like virions and nucleocapsids were also detected at high levels. However, RT-PCR and sequencing proved that the new variant and YHV type 1 were closely related but different viruses (80-85% nucleotide sequence identity) (Cowley, et al. 1999). The new virus was named gill-associated virus (GAV) and is now referred to as YHV type 2.

In Australia, GAV has also been associated with other viruses such as Mourilyan virus (MoV), IHHNV, and spawner isolated mortality virus (SMV), and a disease called mid-crop mortality syndrome (MCMS) in *P. monodon* (Oanh, van Hulten, Cowley and Walker 2011). Besides, GAV was found at very high prevalence (close to 100%) in healthy wild and farmed shrimp populations (Cowley, et al. 2009)(Walker, et al. 2010). It is now thought that these viral infections cause disease in farmed shrimp through a complex interaction between the different viruses. This interaction is difficult to understand and predict, but is responsible for decreased production of *P. monodon* in Australian prawn farms (Munro, Callinan and Owens 2011).
Complicating things even more, another virus had been detected in the years before the discovery of GAV in healthy *P. monodon* in Australia (Spann, JE and RJG 1995). The virus was morphologically indistinguishable from the YHV type 1 and 2, but infected shrimps showed no apparent typically signs of disease. Virions and nucleocapsids were observed only within the lymphoid organs from which the virus was named as lymphoid organ virus (LOV) (Spann, et al. 1995). LOV was regarded as non-pathogenic virus, not associated with disease and mortality. Genomic analysis showed a 98.9% nucleotide identity between GAV and LOV, and thus it was concluded that they were the same virus (Cowley, Dimmock, Spann and Walker 2000a). As a result, LOV was considered as a non-pathogenic relative of GAV (Spann, et al. 1995, Spann, et al. 1997a, Spann and Lester 1997b)(Wijegoonawardane, et al. 2008a).

According to OIE reports, YHD seems to be limited to Asia - India - Pacific regions (OIE, 2009). There is no convincible evidence of YHD outbreaks in the Americas until now (Lightner 2011) though some authors reported that an avirulent yellow head like virus was found in both cultured and wild penaeid shrimp such as *P. vannamei* and *P. stylirostris* in Mexico (De La Rosa-Vélez, et al. 2006), (Castro-Longoria, Quintero-Arredondo, Grijalva-Chon and Ramos-Paredes 2008), (Sánchez-Barajas, Liñán-Cabello and Mena-Herrera 2009). Also, it is likely that YHV was present in co-infections with WSSV during the outbreaks in US and Central American in 1999. However, Pantoja and Lightner argued that the diagnosis of YHLV was most probably due to misinterpretation of histopathological characteristic of the lymphoid organ necrosis caused by acute WSSV infection. (Pantoja and Lightner 2003)

Apart of YHV type-1 and type-2 / GAV, four more genotypes of YHV have been identified in shrimp cultured in Asia (Thailand, Indonesia, Vietnam, India, Malaysia, Taiwan and Philippine) and Africa (Mozambique) (Wijegoonawardane, et al. 2008a). And even though they can be observed at high prevalence, no disease outbreaks have been related to them (Walker, et al. 2010). Because of the complexity of this situation, but the close genetic relation of the different strains, several authors have started to refer to a complex: the ‘yellow head complex viruses’ (YHCV) or ‘yellow head’-like viruses (YHLV) (Wijegoonawardane, et al. 2008a). These six distinct genotypes (YHV type 1 - YHV type 6) are morphologically indistinguishable, but only types 1 (‘classic’ YHD) and type 2 (GAV) are pathogenic, with type 1 being responsible for the majority of the crop losses (Wijegoonawardane, et al. 2008a).
2.4.2. Taxonomy of yellow head complex viruses

Initially, the yellow head like viruses were mistakenly described as baculoviruses due to their size and enveloped rod-shaped appearance (Chantanachookin, et al. 1993). Then, upon the discovery that the genome is ssRNA, it was suggested that the virus was either a rhabdovirus or a coronavirus (Wongteerasupaya, et al. 1995). Later, the virus was classified into the family Rhabdoviridae because the genome was reported to be negative in polarity (Loh, Tapay, Lu and Nadala Jr 1997, Nadala, Tapay and Loh 1997). Tang and Lightner (1999) found by in situ hybridization and sequence analysis that YHV had a positive strand RNA genome. In 2000, (Cowley, Dimmock, Spann and Walker) analyzed the ORF’s in the genome, from which Cowley and Walker (2002b) concluded that the YHC should belong into the order Nidovirales.

Now the complex is classified as the unique species members of Gill associated virus in the new genus Okavirus (based on the virus replication in the shrimp lymphoid or “Oka” organ) of the new family Roniviridae belonging to the order Nidovirales. (Cowley, Dimmock, Spann and Walker 2001, Mayo 2002; Walker et al 2005)

2.4.3. Morphology and properties of YHCV

The YHCV virion is rod-shaped, has an envelope which contains the glycoproteins Gp116 and Gp64, and is about 150-200 nm x 40-60 nm in size. The nucleocapsid is composed of the p20 nucleoprotein, has a helical symmetry with diameter of 20-30nm and surrounds the positive sense single-stranded RNA genome (Cowley, et al. (2001), (2002b), Jitrapakdee, et al. (2003))

Figure 8. Schematic drawing of a YHCV particle (Adapted from Walker and Cowley, 2001)
Yellow head virus replication was found in the cell cytoplasm of shrimp lymphoid organ, gills, haemocytes and connective tissues (Chantanachookin, et al. 1993)(Spann, et al. 1997a)(Tang, Spann, Owens and Lightner 2002). Free virions are also observed in intercellular spaces (Spann, et al. 1997a). The nucleocapsid of YHV becomes enveloped by passage through the endoplasmic reticulum or budding through the plasma membrane. (Duangsuwan et al. 2011)

The complete genome of YHCV was mapped, and found to consist of 26,235 nucleotides with 4 ascertained open reading frames (ORF) (Cowley, et al. 2002b). ORF 1a and 1b encode non-structural enzymes involved in replication and transcription such as helicase, polymerase, exonuclease… (Cowley, et al. (2000b); Sittidilokratna, et al. (2002)) ORF 2 was identified to encode the p20 nucleoprotein (Sittidilokratna, Dangtip, Cowley and Walker 2008). ORF 3 was assumed to encode gp64 and gp116. The function of ORF4 is still unclear.

![Figure 9. Organization of the 26,235 nt (+)ss RNA YHCV genome (Cowley et al, 2002)](image)

2.4.4. Host range and pathogenicity

Only YHV type 1 and 2 (GAV) have been reported to be pathogenic to penaeid shrimp, but GAV is considered to be far less virulent compared to YHV type 1 (Wijegoonawardane, et al. (2008a); Cowley, et al. (2004)). *P. monodon* appears to be most likely the natural host of YHCV because most reports of the virus have been in *P. monodon*, cultured throughout the Asia - Indo Pacific areas, where is its natural distribution. So far, GAV in Australia has only been reported in *P. monodon*, where this species appears to be the only natural host (Walker et al, 2001).
The original source of the YHV type 1 infection in Thailand in 1991 remains unknown. However, some suggest that it might have already appeared earlier and caused serious disease problems in Indonesia, Malaysia, China and Philippine as well as the crash of *P. monodon* industry in Taiwan in 1986-1987 (Lightner, 1996).

It was also hypothesized that YHD in *P. monodon* is a result of cross-species transmission from a natural reservoir species such as metapenaeid, in which no disease occurs (Walker, et al. 2009). This hypothesis was supported by the survey performed by Ma, Overstreet and Jovonovich (2009). The data also showed that daggerblade grass shrimp, *Palaemonetes pugio*, could be a reservoir host for YHV. (Ma, et al. 2009)

Other natural hosts of YHV infections have been reported, including: *P. japonicus, P. merguiensis, P. setiferus, Metapenaeusensisis, Palaemonstyliferus, Euphasiasuperba and Acetes spp.* (Flegel 1997)(Walker et al, 2001) (Longyant, et al. 2006) (Munro and Owens (2007b). Natural infection by YHCV was also observed in *P. esculentus* which was co-cultivated with *P. monodon*. The infection in *P. esculentus* was reported to be chronic.(Spann, McCulloch, Cowley, East and Walker 2003)

Many other penaeid and palaemonid species have experimentally been found to be susceptible to YHCV, although infection and disease susceptibility varied significantly according to the species and age. Certain species of crabs and freshwater shrimp, as well as *Artemia*, have been found to be refractory to YHV infection. (Lu, Tapay, Brock and Loh (1994); Flegel (1997); Longyant, et al. (2006); Ma et al. (2009); Cowley, Hall, Cadogan, Spann and Walker (2002a))

The susceptibility of *P. vannamei* to YHCV infection and disease is not clear. Publications by OIE (2009) and Flegel (2006) mentioned that YHCV is not a major pathogen for *P. vannamei* cultured in Asia. Some reports have shown that *P. vannamei* can be infected with YHCV but disease and mortality were limited (Munro, et al. 2011). It was reported about the YHD outbreak causing mortalities in farmed *P. vannamei* in central Thailand in 2007-2008. The estimated economic loss for those farms was about US$3 million, according to the Thai Animal Aquaculture Association (Senapin, et al. 2010). The original sources of this emergence have been unknown, but unlikely from the post-larvae as they were original from domesticated specific pathogen free stocks. Experimental YHV type 1 infected *P. vannamei* showed signs of disease as early as 2 days post injection and mortality reached 100% within 7 days post injection. (Lu, LM, PC, JA and RB 1995) In American penaeids however, typical signs of yellow head discoloration were not observed during laboratory challenges with
YHV type 1 or 2 (Lightner and Redman 1998). Similarly, YHV type 1 infected *P. vannamei*, in the disease outbreak in 2007-2008 in Thailand, exhibited only faded overall body colour but accumulative mortalities up to 60-70% after 40-60 days grow-out culture (Senapin, et al. 2010). However, histopathological examination also demonstrated typically clinical characteristics of YHD (Flegel 2006).

In 2009, by reverse transcription nested polymerase chain reaction (RT-n PCR), a yellow head like virus was detected in intensive freshwater *P. vannamei* cultured Mexico, with the prevalence of 13% but absences of mortality were reported in these farms. (Sanchez-Barajas et al, 2009)

Other experiments carried out in 4 species of *P. monodon*, *P. japonicus*, *P. esculentus* and *P. merguiensis* showed that different penaeid species have different susceptibility to YHCV infection (Spann, Donaldson, Cowley and Walker 2000). The results demonstrated that *P. monodon* is the most susceptible while *P. japonicas* is the least susceptibility. In several studies, YHV type 1 was found to be highly pathogenic to *P. monodon*, causing 100% mortality within 6 to 14 days after feeding of infected *P. monodon* tissues. ((Spann, et al. 1995, Spann, et al. 1997a); (Vega, Degnan, Hall, Cowley and Wilson 2004); (Cowley, et al. 2000a); (Walker et al, 2001)

Natural and experimental host range for YHV was performed in the following table.
Table 2. Natural and experimental host range for YHV

(Lu et al. 1994; Flegel 1997; Walker et al. 2001; Longyant et al. 2006 and Munro et al. 2007)

<table>
<thead>
<tr>
<th>Species</th>
<th>Evidence of infection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Penaeus monodon</td>
<td>N, E</td>
</tr>
<tr>
<td>Penaeus esculentus</td>
<td>N, E</td>
</tr>
<tr>
<td>Penaeus duorum</td>
<td>E</td>
</tr>
<tr>
<td>Penaeus japonicus</td>
<td>N, E</td>
</tr>
<tr>
<td>Penaeus merguiensis</td>
<td>E</td>
</tr>
<tr>
<td>Penaeus aztecus</td>
<td>E</td>
</tr>
<tr>
<td>Penaeus setiferus</td>
<td>E</td>
</tr>
<tr>
<td>Penaeus stylirostris</td>
<td>E</td>
</tr>
<tr>
<td>Penaeus vannamei</td>
<td>E</td>
</tr>
<tr>
<td>Metapenaeus brevicomis</td>
<td>E</td>
</tr>
<tr>
<td>Metapenaeus enis</td>
<td>N</td>
</tr>
<tr>
<td>Metapenaeus affinis</td>
<td>E</td>
</tr>
<tr>
<td>Metapenaeus bennettae</td>
<td>E</td>
</tr>
<tr>
<td>Palaemon styliferus</td>
<td>N</td>
</tr>
<tr>
<td>Euphausia superba</td>
<td>N</td>
</tr>
</tbody>
</table>

N, natural infection; E, experimental infection.

2.4.5. Transmission of YHCV

Horizontal transmission of YHCV was successfully performed by injection, immersion or digestion of infected shrimp tissues and by co-habitation with infected shrimp (Longyant, et al. (2006) ; Castro-Longoria, et al. (2008)). Walker et al, 2001 announced that occurrence of YHD was related to the viral load. YHCV extracted from healthy infected P. monodon and administered to uninfected shrimp caused infection but no mortality. However when the viral titer in the healthy shrimp reached an equivalent to that in the diseased shrimp, the disease occurred.

There has been no official publication proving vertical transmission of YHCV. However, high levels of YHCV were detected by RT-PCR in spermatophores, seminal fluid and mature ovarian tissue (Cowley et al, 2002). However, nauplii and zoeae were negative for YHV infection. It was suggested that vertical transmission of YHV is associated with the egg.
surface so that most of virus is lost when the larvae hatch. Moreover, Cowley et al, 2002 explained that absence of a developed lymphoid organ - considered as the primary replication site of YHCV, in early life stages of shrimps seemed to limit the potential infection in early larval stages. In spite of this, the identification of lymphoid organ spheroids (LOS) and YHCV particles showed that there was a degree of infection in *P. monodon* postlarvae. (Cowley, et al. 2002a).

![Diagram of infection and disease cycle](image)

**Figure 10.** Model of the infection and disease cycle of the yellow head complex viruses, as proposed by Walker et al, 2001

The high prevalence of YHCV infections in healthy *P. monodon* throughout the Indo-Pacific areas has led to the hypotheses that YHCV must be maintained in the population in chronic, subclinical infections and vertically transmitted (Walker et al 2001; Spann et al, 2003). Moreover, physical stress induced by poor water quality or fluctuations of other environmental factors is thought to play a critical role as trigger for YHD outbreaks (Walker et al, 2009).

**2.4.6. Gross signs**

YHV type 1has been reported to infect cultured *P. monodon* from late postlarval stages onwards, but massive mortalities caused by YHD outbreaks were usually encountered from early to late juvenile stages to sub-adult 5-15 g in size, especially at 50-70 days of grow-out.
culture (Lightner 1996). Natural disease outbreaks have only been reported in *P. monodon* up to 40g in size (Spann et al, 1997; Munro et al, 2007). The first gross sign in *P. monodon*, was a rapid increase in feed consumption during a few days, followed by a sudden cessation of feeding (Chantanachookin et al. 1993). Soon after the onset of anorexia, shrimp start to swim slowly and erratically near the edge of the pond. From 3-5 days after the first observed signs, mortalities rapidly accelerate, often leading to 100% mortality. One typical gross sign in dying shrimp due to YHV-type 1 was a pale-yellow appearance of the cephalothorax and gills, resulting from the discoloration of the hepatopancreas (Chantanachookin et al. 1993); (Cowley, et al. 1999).

![Gross signs of yellow head infection](image)

Figure 11. Gross signs of yellow head infection are characterized with a yellowish discoloration of the cephalothorax region. (Chantanachookin et al, 1993)

The disease outbreaks caused by GAV/YHV-type 2 in *P. monodon* in Australia were reported without typical signs but common development of a reddish appearance, primarily in appendages, tail fan and mouth parts (Spann et al, 1997; Munro et al, 2010). It was also reported that the gills changed to pink color. Fouling of the gills and shell and tail rot were often observed in infected shrimp (Spann et al, 1997). Recently, GAV was found at very high prevalence (close to 100%) in healthy wild and farmed shrimp populations without disease signs. (Cowley, et al. (2000a); Walker et al, 2001, Cowley, et al. (2009); Munro, et al. (2011))

The other four genotypes of YHCV have only been reported in *P. monodon* which showed no apparent signs of YHD or other signs of disease (Walker et al, 2009 & 2010).
According to Lightner (1996), experimental YHV type 1 infections in *P. vannamei* and *P. stylirostris* did not result in typical signs of YHD, although the shrimp did die, they became pale when moribund. A similar result was reported by Tang et al (2002) when juvenile *P. vannamei* (1–2 g) were experimentally inoculated with GAV. The shrimp became moribund after 6 days post infection with GAV (originally from moribund *P. monodon* collected at GAV outbreaks in Australia), but 33% of shrimp did survive up to day 14 post injection. These data showed that GAV can also cause infection and mortality in *P. vannamei*, but the development of disease symptoms was different, and the mortality was less acute.

Chronic infection of YHV was also reported in *P. esculentus* but no cross signs of the disease were observed (Spann, et al. 2003)(Munro, et al. 2007b)

2.4.7. **Target organs of YHCV**

YHCV infections were found in most of organs of shrimp, showing that the infection is systemic (Munro, et al. 2007b). The very first reports of YHD in *P. monodon* from Chantanachookin et al (1993) presented histological evidence of YHV type 1 infection in the lymphoid organ, gills and hepatopancreatic interstitial cells (Chantanachookin et al, 1993). Also, in experimentally YHV type 1 infected, moribund *P. vannamei*, the virus was found in 9 different organs including the gills, nerve cord, lymphoid organ, heart, midgut, hepatopancreas, muscleand eyestalk (Lu et al, 1995). Similarly, Tang, et al. (1999), by using in situ hybridization to detect YHV type 1 infection in *P. vannamei*, observed positive signals in tissue sections of lymphoid organ, cuticular epithelium, gills, connective tissues of hepatopancreas, heart, antennal gland, hematopoietic tissue, nerve tract, midgut cecum and muscle. Presence of YHV in haemolymph further confirmed the occurrence of a systemic infection (Cowley et al, 2000; Spann et al, 2003; Munro et al, 2007).

According to Lu et al (1995), lymphoid organ and gills contained 10 to 800 times higher titers of YHV type 1 than the other tested tissues and organs. As a result of that, it was suggested that LO, gills and HST were the primary target organs for YHV type 1replication. Likewise, the lymphoid organ was considered as the main primary target organ of YHCV by several other scientists (Chantanachookin et al, 1993; Tang et al, 1999 and 2002; Wang CS et al, 1996; Wang and Chang, 2000; Cowley et al, 2001, Khanobdee, Soowannayan, Flegel, Ubol and Withyachumrnarkul (2002); Soowannayan, et al. (2003); Anantasomboon, Poonkhum, Sittidilokratna, Flegel and Withyachumrnarkul (2008); Duangsuwan, et al. (2008a), Duangsuwan, Tinikul, Chotwiwatthanakun, Vanichviriyakit and Sobhon (2008b)).
In GAV infected *P. monodon*, virus replication was found in cell cytoplasm, primarily in lymphoid organ, gills, haemocytes, hematopoietic tissue, connective tissue (Cowley et al, 2001), antennal gland and cuticular epithelium of stomach (Tang et al, 2002). But there was a different distribution of virus depending on chronic or acute infection. As reported by Spann et al (2003), in chronic GAV infected *P. monodon*, there was no evidence of GAV infection in other than in lymphoid organ cells, only in the cell cytoplasm within the spheroid bodies. In contrast, in acute infection, by ISH technique, GAV was detected in the connective tissues of most major organs and throughout the lymphoid organ (Spann et al, 2003). Also, RT-nested PCR detected GAV in male spermatophores at levels significantly higher than that detected in the lymphoid organ (Cowley et al, 2002). This finding supports the possibility that GAV transmits vertically.

2.4.8. Cytopathology

YHCV replication was observed in the cell cytoplasm. By electron microscopy, observation of lymphoid organs from infected shrimp showed that numerous enveloped virions and nucleocapsids scattered or enclosed in the vesicles within the cytoplasm (Wang et al, 2000). The same results were reported by several scientists (Spann et al, 1997; Cowley et al, 2001, Wang et al, 2000; Duangsuwan et al, 2011). But it is still unclear how the viruses are assembled. Spann, et al. (1997b) assumed that the nucleocapsid of YHCV could become enveloped by passage through the endoplasmic reticulum or the virions invading the interstitial spaces of the lymphoid organ gained their envelope by passage through the plasma membrane. Moreover, the process of viral entry into the cell of YHCV is still poor understood. Duangsuwan et al (2011) suggested that the YHCV particles entered the cells by endocytosis, became uncoated and passed into the cytoplasm where the viral genomes were replicated and nuclecapsid proteins synthesized. Then, the nucleocapsid with genomes budded into rough endoplasmic reticulum (RER) and transferred through Golgi compartment where the envelopes were formed. Finally, the completely enveloped viral particles were released by exocytosis at the cell membrane.
YHV type-1 infected cells exhibited hypertrophic nuclei, pyknotic nuclei, and cytoplasmic basophilic inclusions (Chantanchookin et al, 1993; Flegel, 2006). The extensive abnormalities of the lymphoid organ such as nuclear malformation, cytoplasmic degradation and necrosis in the wall of tubules were reported as typical lesions due to YHCV infection (Chantanachookin et al, 1993; Spann et al, 1997 and 2003; Flegel et al, 1997; Tang et al, 2002; Khanobdee et al, 2008; Duangsuwan et al 2008 and 2011). The viral distribution in lymphoid cells (haemocytes and stromal cells) was earliest found by TEM at 18 hours post injection (Duangsuwan et al, 2011). However, the distribution of YHCV in the lymphoid organ varied between individuals (Spann et al, 2003). The lymphoid organ was found exclusively in penaeid shrimp, which was believed to play a central role in immune response against foreign materials and to function as a major phagocytic organ of penaeid shrimp (Duangsuwan, et al. (2008a), (2008b); Rusaini, et al. (2010)).

In addition, the forming of lymphoid organ spheroid was observed in YHV infection shrimps (Spans et al, 2003; (Duangsuwan, et al. 2008b); Rusaini et al, 2010). Due to observation of lymphoid organ spheroid (LOS) during infection of several other shrimps, it was suggested that spheroid formation was indication of a non-specific defensive response ((Hasson, Lightner, Mohney, Redman and White 1999, Anggraeni and Owens 2000)(Duangsuwan, et al. 2008b)(Rusaini et al, 2010). Hypothesis was that spheroids were formed when phagocytes or haemocytes quarantine virus and accumulate in the haemal sinus between tubules. The
spheroid found in natural chronic infected shrimp and in survivors from acute infection suggested that active sequestration of virus exited and this could took an important role in modulation the spread and replication of virus in other tissues (Hasson et al, 1999; Spann et al, 2003). According to Hasson et al (1999), on the one hand, the virus could continue replicate within spheroid, then escape and enter the circulatory system and return to the lymphoid organ where they were isolated by phagocytes and new spheroids were formed. On the other hand, viruses were eliminated by apoptosis if infected cells within spheroid. The infection was cleared resulting in a normal lymphoid organ. Hence, it was suggested that the former process dominated in chronic YHCV infected shrimp. (Spann et al, 2003) Although the spheroids were common observed in viral and bacterial infection, their origin, mode of formation and relationship to lymphoid tubules have been still poor understood.

Apoptosis and necrosis were evident in YHCV infected cells and tissues with pyknotic and karyorrhetic nuclei and densely basophilic inclusions in histological sections. (Spann et al, 1997; Khanobdee et al 2002)

Results of GAV challenging P. monodon were presented that the lymphoid organ was necrotic and disorganized in the stromal matrix of tubule structure from the severe infection (Tang et al, 2002). Khanobdee et al (2002) found chromatin condensation and DNA fragmentation which were considered as characteristics of apoptosis in hemocytes, lymphoid organ and gills of infected shrimp. In acute infected shrimp, 50% of lymphoid organ displayed “gapping” between the tubules due to necrosis (Spann et al, 2003). The gills of disease shrimps displayed damages such as fusion of gill filaments, pyknotic and karyorrhetic nuclei and necrosis. The same pathological features were observed in other organs such as the interstitial tissues of the hepatopancreas, connective tissue underlying the midgut, hematopoietic tissue, heart and so on. (Khanobdee et al, 2002). The extensive deterioration of vital tissues such as hemolymph, gills, lymphoid organ, and heart suggested that many essential functions had been damaged and malfunctioned, which could have led to the collapse of vital functions followed by death (Khanobdee et al, 2002). However, no inflammation was detected in infected shrimp which suggested that cell death caused by YHCV infection may be due to apoptosis rather than necrosis. From the viral accommodation theory proposed by Flegel &Pasharawipas , apoptosis may be related to the phenomenon of tolerance in shrimp to YHCV infection (Anantasomboon et al, 2008) Flegel (2007), Flegel (2009), Flegel and Sritunyalucksana (2011).
2.4.9. Diagnostic methods

The shrimp culture industry needs convenient, viable, trustworthy and cost-effective diagnostic techniques for the major circulating shrimp viruses. The fact that the YHV complex contains at least 6 different genotypes, which are morphologically and histopathologically indistinguishable, presents a problem. Only type-1 and type-2 are known to cause disease, and a positive diagnosis caused by the other types might unrightfully give the farmer the idea that the clinical problems of his shrimp are caused by YHCV. Therefore, the development of specific and sensitive diagnostic tools is an important objective for the disease control.

In general, YHCV infection of shrimp can be recognized by densely basophilic inclusion bodies in gills by H&E staining or by hemolymph smears (Flegel, 2006)

Figure 13. Gill of YHV infected shrimp stained with H&E in normal paraffin sections (A) and in rapidly fixed and stained (3 hours) whole mounts (B). The densely stained purple inclusion can be easily recognized in infected gills. (Flegel, 2006)

Figure 14. Hemocytes from normal and YHV infected shrimp. The disintegration of the nuclei is clearly evident in the YHV infected shrimp (Flegel, 2006)
Transmission electron microscopy (TEM) is considered as the golden standard test for visualization and confirmation of YHV infection. (Munro et al, 2007) However, due to time consuming and inevitable killing of samples, the method seems neither to be helpful for detection at early stage of infection or for screening hatchery broodstocks.

To detect and differentiate 6 genotypes of YHV, especially for 2 virulent and pathogenic genotypes YHV and GAV, antibody ((Nadala Jr and Loh 2000); Sithigorngul, et al. (2000), Sithigorngul, et al. (2002); (Soowannayan, et al. 2003), in situ hybridization (ISH) (Tang et al 1999 and 2002, Spann et al, 2003), conventional RT-PCR (Cowley, et al. (1999), Cowley, et al. (2000a)), real time PCR (Dhar, Roux and Klimpel (2002); Vega, et al. (2004)), reverse transcription nested PCR (RT-nPCR) (Cowley, et al. 2004), and consensus RT-nested PCR (Wijegoonawardane, Cowley and Walker 2008b) have been designed and developed rapidly. The sensitive multiple RT nested PCR (RT-nPCR) have been successfully developed to co detect and distinguish YHV and GAV isolates. (Cowley, et al. 2004) It also reported to be helpful for examining suspected carriers of YHV and testing if they can transfer the virus to farmed shrimp (Cowley et al, 2004) (Flegel 2006).

In 2010, a consensus real-time RT-PCR for detection of all genotypic variants of yellow head virus of penaeid shrimp was developed. The technique showed it to be 99.8% efficient and capable of detecting as few as 2.5 RNA copies reliably. As the test detects all six YHV complex genotypes and is extremely sensitive, capable of quantifying infection loads, it should prove useful for managing infections in shrimp hatchery broodstock. (Wijegoonawardane, Cowley and Walker 2010)

Although the PCR techniques have proved themselves as highly sensitive for detection of YHV, there are still limitations for their widespread application such as requirement of special equipment, expensive reagents and well trained personnel (Munro et al, 2007)

In situ hybridization using DNA probe targeted to regions in the ORF1 gene have allowed to cross detection of YHV type 1 and GAV (Tang et al, 1999 and 2002) However, the problem is that the viral RNA is very labile and easy to be damaged during specimen preparation, especially in a fixation step with Davidson’s fixative (Flegel, 2006). According to Flegel (2006), Dr Walker’s group has successfully developed a preservative solution (containing of 80% ethanol, 20% glycerol and 0.25% mercapto-ethanol) for field samples. With it, the samples are kept in reasonable length at room temperature.

In addition to nucleic acid based detective methods for YHCV, antibody based tests such as immunohistochemistry (IHC), dot blot assay and lateral flow chromatographic assay are
widely used. Being highly sensitive and specific, providing quick but accurate results, these techniques are ideal for on farm screening of disease and pathogen if using correctly. (Munro and Owens 2006) Immunological tests using monoclonal antibodies (MAbs) to a surface glycoprotein or nucleocapsid protein of YHV have recently detected and differentiated of YHV and GAV (Sithigorngul et al, 2000 and 2002; Soowannayan et al, 2003). Polyclonal antibodies (PAbs) and specific MAbs against GAV have been rapidly developed for detection of GAV by enzyme linked immunosorbent assay (ELISA) (Munro and Owens 2007a).

The lateral flow chromatographic assay - so called “strip test” is interesting because it is rapid result, cheap, convenient and simple to use at pond-side by farmers (Sithigorngul, et al. 2007) and (Sithigorngul, Rukpratanporn, Chaivisuthangkura, Sridulyakul and Longyant 2011)
In 2005, Munro and Owen (Munro and Owens) developed a quantitative, low cost test based on haemagglutination (HA) using chicken erythrocytes to detect the viral load of GAV. Comparison with RT-nPCR and ELISA, it is suggested that HA has been a useful alternative for on farm detection of GAV (Munro et al, 2005, 2006 and 2007).

2.5. Current viral disease management strategies

Strategies for health management in shrimp are based on the preventive principles of pathogen exclusion and elimination, avoidance of stress factors and improvement of organism immunity because shrimps, as invertebrates, lack the vertebrate adaptive immune response that provides a mechanism for protection and application of vaccination against viruses. (Flegel et al, 2008; (Flegel 2012); (Walker et al, 2009); Lightner (2011), Lightner, et al. (2012))

2.5.1. Specific pathogen free (SPF) and specific pathogen resistance (SPR) shrimp stock

The high quality seed plays a critical role in reducing the risk of crop failure. Thus SPF and SPR stocks have brought to the shrimp culture industries far more advantages in better health management, production improvement and sustainable development than ever before.

In the past, using wild caught postlarvae or ones produced in hatcheries from wild caught broodstock has caused problems related to diseases and disease spread, which was blamable for crashes or reductions of the sustainable development of many shrimp culture industries (Flegel 2006) (Lightner 2011a). To improve the situation, shrimp stocks which are known to be free of the major pathogens have been commonly practiced in most shrimp culture
regions. Specific pathogen status refers to the absence of specific pathogens from a population of shrimp. The SPF shrimps are better than non - SPF ones by ensuring that pathogens (especially major viruses) are not present on shrimps at the start of grow-out. This brings a great advantage for keeping the pathogen out of production systems or below the levels of causing disease. (Moss, Arce, Otoshi, Doyle and Moss 2007, Moss, Moss, Arce, Lightner and Lotz 2012) (Lightner, 2011a)

The development of specific pathogen free domesticated stock of *P. vannamei* was initiated in the US by US Marine Shrimp Farming Program (USMSPF) in early 1990s. The current list of specific pathogens has had 10 major viruses, in which seven one have been notified by OIE, including WSSV, YHCV, TSV, IHHNV, HPV, BP, MBV, BMN, IMNV and PvNV) as well as certain parasites and bacteria. (Lightner, 2011)

Thanks to the program and widespread cultivation of the species, the global cultured shrimp industry has performed considerably sustainable increases in production and value. The global shrimp production has tripled over the last decade. (Wyban, 2009)

![Figure 17. World cultured shrimp production (thousand tonnes) was doubled from 1999 to 2006 and percent contribution of SPF *P. vannamei* has increased from 10% to 75% (Wyban, 2009)](image)

The domesticated SPF stocks have been also available for *P. stylirostris* and being developed for other shrimp species, such as *P. chinensis* and *P. monodon*. Recently, domestication and selective breeding of *P. monodon* in Australia have achieved promising successes. Stocks of *P. monodon* reared for five generations have had a 10 fold higher nauplii production than first generation broodstock.

Shrimp breeding and selection for resistance to a specific pathogen have achieved success in *P. vannamei* against Taura syndrome virus (TSV) and in *P. stylirostris* against infectious hypodermal and hematopoietic necrosis virus (IHHNV) (Lightner, et al. 1998); (Moss, et
An outstanding example was in US, within 5 year (1998 - 2003) after using stocks against to TSV, the shrimp production has significantly increased from 1500 tonnes to 6150 tonnes (Wyban, 2009). TSV resistant SPF/SPR stocks have been recent reported to reach survival rate of 80-100% in laboratory challenge with four known TSV strains (Lightner, 2011a). But, the “specific pathogen resistant” terms do not mean that the shrimp resist to infection but that they do not become diseased after infection. Also, the fact is that the shrimp selected of tolerance for one pathogen does not seem to be resistant to another Moss, et al. (2007), Moss, et al. (2012). Understandings about mechanisms of viral resistance as well as genetic information have been essential for an effective selection program to develop the disease resistance of shrimp. Unfortunately, up to now, the development of shrimp stock resistant to WSSV, the most virulent pathogen, has got very little successful (Cock, Gitterle, Salazar and Rye 2009).

In the near future, we can expect to see other advantages to come such as real disease resistance, fast growth and stress resistance of new selective shrimp strains.

Also, to support the development of selection and breeding of shrimp and to protect the broodstock’s right, triploid induction has been applied in four shrimp species: Ferrenopeaneus chinesis, P. japonicus, P. monodon and P. vannamei. The standard protocol has been still further developed but the initial research has performed promising results. (Sellars, Arce and Hertzler)

### 2.5.2. Better management practice (BMP)

To develop and implementation of practice, affordable and effective measures to reduce disease and environmental impacts, Better management practices (BMP) have developed for small holder farmers. BMP have provided guidance designed to increase efficiency productivity and profitability by reducing the risk of shrimp healthy problems and diseases, decreasing the environmental impacts, improving food safe and product quality and increasing sustainable development. (Walker et al, 2009)

### 2.5.3. New diagnostic methods

The availability of easy, convenient, rapid and reliable detection methods takes an important role in health management and disease outbreak control. The most rapid and sensitive PCR technique have been introduced, which takes about 3 hours to complete a test (Flegel, 2006).
Proteomics technologies have also played a major role due to assistance both in the development of new vaccines and diagnosis of diseases. (Rodrigues, Silva, Dias and Jessen) However, most of current trustworthy detective methods, such as PCR, are quite expensive, complicated and designed for highly trained people. Unskilled farmers need devices far more easily to use but still reliable enough. To meet these requirements, flow chromatographic immunodiagnostic strips based on labeled monoclonal antibodies have been designed and being commonly used due to highly specific, reasonably priced and so user friendly, which can be used pond-side by farmers. The strip test of YHCV using monoclonal antibody Y19 against the p20 nucleocapsid protein has been developed and available in 2007. The kit provides beneficial features such as simple, convenient, and rapid results without requirements of complex tools and special skills. (Sithigrongul et al, 2007)

The core remain problem of PCR as well as DNA based detective methods are unable to detect a new emergent pathogen without specific designed probes. Hence, new cost-effective and user friendly detection methods are essential to be urgently designed and developed. (Flegel et al 2008)

2.5.4. Microbial intervention and immunity improvement

2.5.4.1. Probiotics

Probiotics - “bio friendly agent’ have been common used in aquaculture in general, and in shrimp culture in particular (Mohapatra, Chakraborty, Kumar, DeBoeck and Mohanta 2012). The term “probiotics” was derived from the Greek words “Pro” and” bios” which mean “life”. Probiotics has defined as live micro-organisms which are administered in sufficient amounts to give a health benefits on the host (FAO, 2001) or introduced into the culture environment to increase water quality and to control pathogens by competitive exclusion and antimicrobial activities. (Balcázar, et al. 2006); (Mohapatra et al, 2012)

Beyond these advantages, for shrimp culture, probiotics are well knows as playing important role in improving the innate immunity. Due to lack of adaptive immune responses, vaccination seemed to be so far successfully applied in shrimp. Probiotics have proved themselves as a great alternative to help the shrimp fighting against with pathogens as well as stress factors ((Balcázar, et al. 2006)). For example, the use of Bacillus sp strain 11 was reported providing protection by activating both cellular and humoral immune defenses of P. monodon (Rengpipat, Rukpratanporn, Piyatiratitivorakul and Menasaveta 2000). Likewise, the supplement of Bacillus subtilis E20 (10^9 CFU/ml) into the larval rearing water of P.
vannamei showed a significant improvement of larval survival and development, stress tolerance and immune status. Also, remarkable increases of phenoloxidase and phagocytosis activities were observed in shrimp larvae (Liu, Chiu, Shiu, Cheng and Liu 2010). Immunity enhancement in shrimp by probiotics was also published by other scientists such as Gullian, Thompson and Rodriguez (2004) and Zokaeifar, et al. 

Antiviral activity is also highlighted as a great advantage of probiotics. Some probiotics have been reported to have antiviral effects. Strains of Pseudomonas sp., Vibrio sp. and Aeromonas sp. performed antiviral activities against IHHNV and Bacillus spp. also reported positive effects against to WSSV. ((Bachère 2003); (Balcázar, et al. 2006)

In addition, probiotics have proved to have no undesirable side effects to aquatic organisms, even by oral administration. (Mohapatra et al, 2012)

Probiotics play a vital role in health management for sustainable shrimp culture development, especially currently chemotherapeutic agents have been restricted due to public concerns of antibiotic resistance and ecological effect. Probiotics has promised proved advantages: appetite and growth enhancement, disease prevention, stress reduction, and costly chemotherapy saving, especially antibiotics. (Mohapatra et al, 2012) Therefore, knowledge of the mode action of candidate probiotics is highly essential. More detailed and focused researches are necessary for betterment of future shrimp culture. (Flegel et al. 2008; Walker et al, 2009)

2.5.4.2. Immunostimulants

Immunostimulants are chemical substances that activate white blood cells (leukocytes), hence, render animals more resistant to infectious diseases, reducing the risk and loss of disease outbreaks (Raa, 2000). In shrimp, hemocytes have functions as macrophages, granulocytes and natural killer cells in vertebrates, which are responsible for antimicrobial products, phagocytosis, and removal of foreign particles. Due to lack of specific immune system, shrimp mainly rely on the innate immune system to fight with pathogens (Bachère 2000). Hence, immunostimulants play key roles in shrimp against pathogens, especially viruses. (Dalmo and Bøgwald 2008); (Zhao, Jiang and Zhang 2011)) Immunostimulants can be crude preparation of whole dead microbial cells (bacteria or yeast), cellular components of bacteria, fungi and yeasts, purified products from plants and microbes, pure chemicals. (Raa, 2000; Flegel et al, 2008)

Laminarin, barley, glucan, lactoferrin, levamisole, lipopolysaccharides, curdlan, scleroglucan, zymosan, chitosan, β-glucans, dextran, lentinan, krestin, saponins, herbal
extracts, peptidoglycans and so forth, are common used in aquaculture. (Maqsood et al, 2011; Ringo et al 2012)

2.5.4.3. Herbal immunostimulants

Natural plant products have presented a viable alternative to antibiotics and other banned drugs due to being safer to aquatic animals and environment. Herbals and herbal extracts can be used in aquaculture not only as remedies but as growth promoters, stress resistance stimulators and preventatives of infections (Citarasu 2010). Many studies of immune-stimulation and antimicrobial activities of herbal products in shrimp species have been published. Direkbusarakom et al (1995) used extraction of *Phyllanthus spp.* (*P. amarus, P. debilis, P. pulcher, P. reticulatus* and *P. urinaria*) to inactivate YHV in *P. monodon*. The virucidal activities of these extracts were tested by mixing the virus with plant extracts followed by incubation and injection into healthy shrimp. Antiviral activity was assessed by observation of the mortality rates of the injected shrimp. Extracts of *P. amarus* and *P. urinaria* provided the highest survival rate of 80-100% of YHV infected shrimp while the untreated challenged groups died 100% within 10 days. The extracts also enhanced phagocytic activity in black tiger shrimp.

The same scientists also reported that *P. monodon* fed with extract of *Clinacanthus nutans* had 95% survival rates when exposed to yellow head virus (YHV) compared to only 25% of survival in control group (Direkbusarakom et al., 1996). Administration of extracts of twenty medicine plants, such as *Cyanodon dactylon*, was reported to enhance the immunity of shrimp and efficiently protect the shrimp against WSSV infection. (Balasubramanian, Sarathi, Kumar and Hameed 2007); (Yogeeswaran, et al. 2012)

2.5.4.4. Shrimp “vaccine”

In Kuruma shrimp survivors from a WSSV outbreak in 2000, a factor, called “a quasi-immune response“, was discovered in shrimp hemolymph related to prevent shrimp from dying caused by WSSV infection. (Venegas et al, 2000) Understanding of the quasi-immune response is still poor however it is true that the shrimp are protected from disease but not from infection. From the report, several studies have been carried out to exam the possibility of protecting shrimp from viral pathogens by using “vaccine like substances” or tolerines. (Flegel et al, 2008b)(Flegel, et al. 2011)

A commercial product with trade name as SEMBVAC consisting whole particles of WSSV has been introduced. The vaccinated shrimp was reported tolerance to WSSV and sufferance less from disease (Bright Singh et al, 2005; Flegel et al, 2008). Other tolerines consist of
single or mixture of protein subunits of viral particles have been developed rapidly however most of publications have focused on vaccination against WSSV for common cultured shrimp species such as *P. monodon, P. vannamei, P. chinensis, M. japonicas* and crayfish. (Witteveldt, Cifuentes, Vlak and van Hulten 2004)(Haq, Vignesh and Srinivasan 2012)

Recombinant WSSV envelope proteins (VP26, VP28, VP19, VP292 or VP466) have been used to design vaccines against WSSV and achieved successes, more or less. Particularly, VP28 gene was used to design DNA vaccine. The results of vaccination trials showed a significantly higher survival rate (56.6 - 90%) in vaccinated shrimp with VP28 when compared to control groups (100% mortality). The vaccinated shrimp showed significantly high level of prophenoloxidase and superoxide dismutase when compared to the control groups. The increase of innate immune response activities might be responsible for developing resistance against WSSV in DNA vaccinated shrimp (Kumar, Kawai and Akira 2011). However these vaccines provide such a short term protection. (Flegel et al, 2008; Walker et al, 2009) (Johnson, van Hulten and Barnes 2008)

A rare study on recombination protein vaccination in *P. monodon* against GAV - RNA virus was carried by a group of Australian scientists in 2010. However, protein vaccination failed to protect the shrimp against GAV infection. The mortality was no significantly different between vaccinated groups and control ones. (Underwood, et al. 2010)

Recently, it was found that a phagocytosis activating protein (PAP) gene could be able to activate phagocytosis of shrimp hemocytes and the immune cellular response of shrimp against pathogens. Experiments were conducted of intramuscular injection bacterially expressed PAP (PAP-phMGFP) into *P.vannamei* challenged by WSSV. The results presented the highest level of PAP and proPO (prophenoloxidase) after 7 days post immunization performing the highest survive rate of 86.61%. Also, this immunization protected shrimp from other pathogens: YHV and *V. harveyi*. Unfortunately, no protection was observed after 30 days post injection. (Khimmakthong, Deachamag, Phongdara and Chotigeat 2011) However, up to now, no commercial product of protein based vaccine is available yet. (Haq, et al. 2012)

2.5.4.5. **RNA interference (RNAi)**

The development and application of nucleic acid base therapeutics for viral infection in cultured shrimp has showed significant promising as an efficient strategy for health
management and disease control. (Shekhar et al, 2009; (Hirono, Faguta, Kondo and Aoki 2011)(Bartholomay, Loy, Dustin Loy and Harris 2012)(Labreuche and Warr)

RNAi was first discovered in the nematode Caenorhabditis elegans (Hannon, 2002) then later in several other organisms. This is a critical pathway used by different organisms, such as arthropods, in gene expression regulation as well as in antiviral defense mechanism. RNAi is identified as a response to existence of double stranded RNA (dsRNA), resulted in sequence specific silencing of a target gene. RNAi technology has recently applied experimentally by injection of dsRNA or short interfering siRNA homologous to viral mRNA to efficiently protect shrimp from viral pathogens: YHV (Tirasophon, Roshorm and Panyim 2005, Tirasophon, Yodmuang, Chinnirunvong, Plongthongkum and Panyim 2007)(Yodmuang, Tirasophon, Roshorm, Chinnirunvong and Panyim 2006), WSSV (Xu, Han and Zhang 2007), TSV (Robalino, et al. 2007), Penaeus stylirostris denso virus (PstDBV) (Ho, Yasri, Panyim and Udomkit 2011) and IMNV (Loy, et al. 2012)

It was reported that intramuscular injection of bacterially expressed dsRNA was effective in protection P.monodon from GAV while oral delivery method was ineffective. Due to the RNAi response against GAV by injected dsRNA targeted to multiple genome sites, it was suggested that this strategy can apply in enhancing protection against other single stranded RNA viruses in broodstocks. (Sellars, M, SJ, NM and JA 2011)

Therefore, shrimp innate immunity enhancement induced by dsRNA or siRNA targeting to specific viral sequences, especially replication genes, is effective and advantaged in therapeutic treatment (Walker et al, 2009). However, remain obstacle to application of RNAi technology is practical, cost-effective delivery systems for commercial scales. (Bartholomay, et al. 2012)

2.5.4.6. Antimicrobial peptides

Up to now, more than 400 antimicrobial peptides were found in both prokaryotic and eukaryotic organisms, in which ≥ 50% comes from insects. Antimicrobial peptides are mostly cationic molecules and have small molecule weight (≤ 10kDa). In shrimp, antimicrobial peptides have been isolated from the haemocytes (Bachère 2003). The production of these peptides is induced in shrimp after they are infected by microbes as innate immune response as antibacterial, antifungal and antiviral agents (Bachère 2000, Bachère, Destoumieux and Bulet 2000,Bachère 2003). Member of the Pennaeidin family have been identified in P. vannamei and several other species of penaeids (Bachère, et al. 2000). It is suggested that the dual functions of penaeidins (chitin binding and antimicrobial
activity) are important in shrimp protection and wound healing, especially for molting stages (Bachère, et al. 2004). Due to antimicrobial activities, specific way of induction and their biological properties, antimicrobial peptides are potential for a new type of therapeutic agents and disease control methods. Being natural shrimp compounds and already present in shrimp, using antimicrobial peptides could not lead to the development of pathogen resistance. Bachère (2003); (Flegel et al, 2008)

In addition, other kinds of proteins are assumed to be related in shrimp immune response to viral pathogens, such as C type lectin and interferon like protein. Understanding of these substance and their activities may lead to development of novel disease prevention methods for shrimp culture. (Flegel et al, 2008) (Flegel, et al. 2011)

2.5.5. Host - virus interaction

Understanding of the relationship between shrimp and viral pathogens is still poor, but progress is being made, both on molecular and genetic level. Evidence exists that viruses, such as WSSV and YHCV, can trigger massive apoptosis (programmed cell death) which leads to shrimp death (Sahtout et al, 2001; Khanobdee et al, 2002; Wongprasert et al, 2003).

If the trigger for this process can be identified, there is a good chance that blocking it can prevent shrimp death and reduce impacts of disease. (Flegel et al, 2008)

One phenomenon in shrimp that is commonly observed is that they can tolerate single and multiple viral infections without gross or histological signs of disease. (Flegel et al, 2004)

The hypothesis of active viral accommodation in shrimp was first proposed by Flegel and Pashawarwipas in 1998, but up to now, the mechanisms are still poor understood. Accommodation is characterized by the presence of an active mechanism to tolerate a pathogen and by the absence of an active defence against it. Recent research data suggested that such viral persistent infection could result in “a specific memory” that somehow reduce the severity of disease by preventing viral triggered apoptosis. Flegel (2007), Flegel (2009), Flegel, et al. (2011) Protection from a viral pathogen by infection of single or multiple other viruses have been investigated by several scientists. Tang et al, 2003 reported about the protection against WSD by persistent IHHNV infection in P. stylirostris. Likewise, protection from YHV infection in P. vannamei pre-infected with TSV was recently published (LF, KFJ and DV 2012). As a result of this, it is suggested that there is an existence of viral interference effects between TSV and YHV which could the sensible reason for the absence of YHV in the Americas. Hence, deeper knowledge of the molecular mechanisms behind shrimp tolerance to viral infections is essential to lead to a better
understanding of host-viral interaction and to the development of novel methods for viral disease control. (Flegel et al, 2008 and 2011)

2.5.6. Using ecological factors to reduce viral disease impacts

As in all systems, the relationship between environmental stress, immunological vitality and the virulence of viral pathogens is fundamental in shrimp culture (Flegel et al, 2008). De la Vega de la Vega, Hall, Degnan and Wilson (2006) reported that short-term hyperthermic treatment of P. monodon augmented expression of heat shock protein 70 (HSP70), a key indicator for environment stress events, and reduced GAV replication. Heat shock proteins have been found to be involved in the inhibition of viral replication and resistance to disease by functioning as molecular chaperones as well as mediating the humoral and cellular innate immune responses. The potential of the strategy to influence viruses in shrimp by manipulating the water temperature is further strengthened by the publication of several studies showing a similar protection against WSSV by high temperature (≥32°C) (Granja, Aranguren, Vidal, Aragón and Salazar 2003); Rahman, et al. (2007a), Rahman, et al. (2007b)(Reyes, Salazar and Granja 2007); (WONGMANEEPRATEEP, et al. 2011).
CHAPTER 3 – MATERIAL AND METHODS

Time and location

- The study was conducted between February and August 2012.
- Experiments and sample analysis were carried out in Laboratories of Virology and Morphology, Faculty of Veterinary Medicine, Ghent University.

Experimental animals

(Lito)penaeus vannamei were imported from Shrimp Improvement Systems, Florida, US. They were certified to be specific pathogen-free (SPF) by the USDA, Miami for the following pathogens: WSSV, YHLV, TSV, IHNV, BP, MBV, BMN, IMNV, Microsporidians, Haplosporidians and NHP bacteria. The shrimp arrived in postlarval stage 10 and were reared in the recirculation system at the Laboratory of Aquaculture and Artemia Reference Center. During rearing water temperature was maintained at 27 ± 1°C, salinity at 35 ± 1 mg l⁻¹ and pH at 7.8 – 8.1. Total ammonia was kept below 0.5 mg l⁻¹ and nitrite below 0.15 mg l⁻¹. The room was illuminated 12 h per day by dimmed TL-light. During the first week, shrimp were fed twice a day with Artemia nauplii. After 3 weeks they were weaned onto commercial pelleted shrimp feed (CreveTec Grower which contents 36.9 % crude protein, 9.0 % lipid, 3.0 % crude fiber, 10.0 % ash and 1.0 % P from Aqua Bio Joosen-Luyckx, Belgium), at a total rate of 5 % of their mean body weight. Before the viral challenge experiments, shrimp were transported to the Laboratory of Virology, Faculty of Veterinary Medicine, where experiments were carried out under biosafety conditions.

Virus

The AUS-96-Ref strain of GAV was kindly donated by Prof. Dr. Peter Walker from CSIRO, Australia. This mother stock was originally isolated from naturally infected P. monodon (Spann et al., 1997) and passaged once in P. monodon (Peter Walker, personal communication).
Once the virus had arrived to our laboratory, it was injected in SPF *P. vannamei* (n = 25, weight=20 g) for the production of a virus stock. For this, the GAV mother stock was diluted 10^{-1} in phosphate-buffered saline (PBS) with pH 7.4 and 50 µl of the dilution was intramuscularly (IM) injected into *P. vannamei* between the 3rd or 4th abdominal segment with an accurate syringe (P/N: 81001/00, 1710 LT, 100 µl, Hamilton Bonaduz) mounted with a 25 gauge needle.

Injected shrimp were kept at 27±1°C in 50 l glass aquaria equipped with water heaters, mechanical filters (Eheim classic 2213, Germany) and continuous aeration. Artificial seawater at salinity of 35 g l^{-1} was prepared with artificial sea salt (Instant ocean, Aquarium systems, France) dissolved in distilled water. The aquaria were covered with glass plates to prevent contamination and shrimp jumping out. The shrimp were fed a restricted diet of 4 pellets twice per day to maintain water quality. The water quality was monitored regularly by measuring ionized ammonia (NH_{4}^{+}) using test kits.

After allowing viral replication for 5 days, infected shrimp carcasses were collected and frozen at -70°C. Then, the presumed GAV-infected shrimp were thawed and the cuticle of carapace was removed using forceps. Collected tissues were placed in a petri dish and kept on ice before being minced finely with scalpel blades and weighed. The tissues were homogenized in PBS (in a volumetric proportion of 1:10) by an ultra-turrax at 5000 rpm, in a container introduced in a box of ice to avoid warming up of the suspension with the danger of inactivating the virus. To remove debris from the suspension, the homogenate was centrifuged at 5500Xg for 20 minutes in 4°C using a swinging bucket rotor. The supernatants were collected and pooled together in a new cold bottle. The pooled supernatants were homogenized and filtered through a 0.45µm membrane into a new, cold and sterile bottle. Finally, the filtered stock was aliquoted and frozen at -70°C.

**Monoclonal antibody (MAb) for the detection of YHCV**

MAb Y19 against the nucleocapsid protein P20 of YHCV (Sithigorngul et al, 2000 and 2002) was kindly provided by Prof. Dr. Paisarn Sithigorngul from the Department of Biology, Faculty of Science, Srinakharinwirot University, Bangkok, Thailand.
**Preliminary test**

In order to check whether the GAV virus stock production and antibody staining for GAV were successful, an inoculation with the newly produced GAV stock was first tested in a limited number of animals. The stock was diluted 1:3 in phosphate-buffered saline (PBS) pH 7.4 and injected IM into 12 SPF *P. vannamei*. Groups of 3 inoculated shrimp were kept at 27±1°C in 50 l aquaria and maintained as described for stock production. Shrimp were collected at 168 hours post injection (hpi) and processed for paraffin sectioning, H&E and immunohistochemistry staining (IHC) (Figure 18).

**Inoculation**
- Route: intramuscular injection
- Dose: 1:3 dilution of GAV stock in PBS
- PBS only for control shrimp
- Volume: 50µl per shrimp

**Experimental conditions**
- 3 shrimp per 50 l tank
- Temperature: 27±1°C
- Salinity: 35g l⁻¹
- Continuous aeration and mechanical filtration
- Commercial pellet: 4 pellets/shrimp; twice per day

**Sampling**
- at 168 hpi

**Fixation**
- Davidson's for 48 h
- Ethanol 50% for 24 h

**Processing**
- Processing, paraffin embedding and sectioning

**Staining**
- H&E staining and Immunohistochemistry

**Aim 1:**
- Check if the GAV stock is infectious

**Aim 2:**
- Check if the antibody Y19 works

**Figure 18.** Experimental plan of the preliminary test to check whether the GAV virus stock production and antibody Y19 staining were successful.
**Time course pathogenesis study of GAV infection in *P. vannamei***

In order to follow-up the spread of GAV infection in time, an experiment was designed in which SPF *P. vannamei* were collected for immunostaining at different time points after injection with GAV (Figure 19). By the time of this experiment, *in vivo* titration experiments had shown that our GAV stock had a geometric mean infectious titer of $10^{5.23}$ SID$_{50}$ ml$^{-1}$. This stock was diluted in PBS to an inoculation dose of $10^3$ SID$_{50}$ for each shrimp. This was injected in a volume of 50 µl between the 3$^{rd}$ or 4$^{th}$ abdominal segment.

Ten negative control shrimp were also included in the experiment and inoculated with 50 µl of PBS. Five virus-injected shrimp were collected for IHC and H&E at 0, 6, 12, 18, 24, 48, 72, 120, 168, 240 and 336 hpi. Five PBS-inoculated shrimp were collected for IHC and H&E at 72 and 336 hpi.
Each inoculated shrimp was kept individually in a 10 l aquarium, filled with artificial seawater at salinity of 35 g l\(^{-1}\), at 27±1°C and with continuous aeration. The aquaria were covered with Plexiglas plates to prevent shrimp jumping out and contamination. The shrimp were fed a restricted diet of 4 pellets per day to maintain water quality. The water quality was regularly monitored by measuring ionized ammonia (NH\(_4^+\)) using test kits, and water changes of 50% were performed when ammonia levels passed 0.5 mg l\(^{-1}\).
**Tissue processing and microtomy**

In order to obtain paraffin sections for H&E and IHC staining, the methodology described by Bell & Lightner (1988) and Lightner (1996) was followed. Collected shrimp were fixed with Davidson’s fixative. To ensure fast fixation, 10% of body volume of fixative was injected in the cephalothorax and tail, and a ventral incision through the cuticle was made to facilitate the entry of fixative. The shrimp were submerged in Davidson’s fixative (10X volume of tissues) for 48 hours. After fixation, the samples were transferred into ethanol 50% until processed for paraffin embedding. After processing and embedding in paraffin, paraffin blocks were trimmed until the tissues had been reached. The blocks were then floated in formic acid for 15 min in order to decalcify the cuticle and reduce artifacts due to cutting. Sections (5 μm) were cut with a microtome and landed on a 50% ethanol bath at room temperature. Next they were moved to a distilled water bath at 46°C using uncoated glass slides. Once the sections were totally stretched on the water surface, they were fished up on silane-coated slides (A3648, Sigma-Aldrich, USA) and left to dry. Slides were finally heated on a warm plate for 1 h at 60°C and placed at 37°C overnight to ensure good attachment of the tissues sections to the slides. The next day they were either stained, or stored at room temperature.

The entire cephalothorax of each shrimp was parasagittally sectioned.

**H&E staining**

H&E stainings were made by using the Linear Stainer II SAKURA automatic staining machine. The slides were passed through steps of xylene, a series of ethanol and water (100%, 95%, 80%, 75% and 50%) to deparaffinize and rehydrate the tissue. The slides were then stained with the nuclear dye (hematoxylin) and rinsed, then stained in the counterstain (eosin). They are then rinsed and run in the reverse manner (taken back through water, alcohol, and xylene) to dehydrate. It took about 1 minute 45 seconds for each step. After mounting with DPX, the slides were ready to observe by microscopes for detection pathological changes in tissues and cells of the shrimp.

**Immunohistochemistry (IHC)**

GAV infection in shrimp was analyzed by IHC according to the protocol of Sithrigorngul et al (2000) and Escobedo-Bonilla et al (2007). The first step was started with putting the slides including a positive control at 55°C for 30 minutes. Sections were deparaffinized and
rehydrated by placing in xylene, ethanol 100%, 95%, 70% and 50% in 5 minutes for each step. Then the slides were washed by Tris+NaCl in 5 minutes. The endogenous peroxidase was blocked by incubating the slides for 30 min at room temperature in a solution of 1% sodium azide and 0.02% hydrogen peroxidase in Tris buffer pH 7.4. Sections were incubated for 1 hour at 37°C with a 1:200 dilution of primary antibody Y19. After washing with Tris+NaCl buffer, the slides were incubated with a 1:200 dilution of biotinylated sheep anti-mouse IgG antibodies (RPN1001 Amersham Biosciences, UK) for 1 hour at 37°C. The slides were washed with Tris+NaCl buffer again before being incubated with 1:200 dilution of streptavidine biotinylated horseradish peroxidase complex (RPN1051 Amersham Biosciences, UK) for 30 minutes at room temperature. Colour development was made with 0.01% of 3, 3' - diaminobenzidine (DAB) (D8001 Sigma-Aldrich, Germany) in Tris buffer without NaCl for 10-15 minutes. The slides were counter-stained with Gill’s hematoxylin and then washed, dehydrated, cleared and mounted. The slides were observed by microscope for detecting target organs of GAV infection and virus quantification. Positive reactions for GAV were visualized by a brown coloration against the blue color of the hematoxylin.

**Quantification of GAV-infected cells**

GAV-infected cells were counted on IHC-stained slides by light microscope (Leica DM RBE, Germany) at a magnification of 400X.

Previous studies quantifying virus infection in shrimp in our laboratory were based on counting individual infected cells (Escobedo-Bonilla et al., 2007; Rahman et al., 2007). However, in the present study, we found GAV positive signals diffusely spread throughout tissues and in large agglomerations of difficult to distinct cells. Hence, we designed an alternative method that quantified GAV infection as the area of positive signal per mm². We counted this area in the lymphoid organ and expressed it as a percentage of infected area of the total organ.

The collected data were analyzed by Microsoft Office Excel 2010.
CHAPTER 4 - RESULTS

4.1. Preliminary experiment

4.1.1. Clinical signs of GAV disease

After completion of the in vivo titrations, it could be calculated back that -with a dilution of 1:3 of the stock- shrimp had been injected with a dose of $10^{3.52} \text{SID}_{50}$ of AUS-96-Ref GAV. With this dose, all challenged *P. vannamei* shrimp remained alive until 7 days post inoculation. GAV-infected animals did not show typical clinical signs of YHD with yellowish discoloration of the cephalothorax or reddish coloration of the body.

Disease symptoms which were observed in GAV-infected animals included: anorexia, soft shell and pale color of the cephalothorax (Figures 20 and 21). However, these signs were only recorded in 10% of the inoculated animals. Often, these animals would be sick during the first few days of the experiment, recover and start to eat again after a few days.

![Figure 20](image)

Figure 20. Upon GAV injection, about 10% of infected shrimp (above) were anorectic with obviously empty midgut in comparison with control shrimp eating normally (below). Also note the pale, opaque coloration of the infected animal, compared to the transparent aspect of the normal shrimp.

IHC analysis of all GAV-inoculated shrimp in the preliminary test showed severe infections in most of organs. These shrimp were used as a positive control for the histopathology and IHC stainings in the time course study.
4.2. Time course pathogenesis study of GAV infection in *P. vannamei*

During the 2-week time course study, no disease and mortality were observed in the shrimp.

4.2.1. Determination of the target organs of GAV in *P. vannamei*

By IHC, GAV positive signals were observed in lymphoid organ, gills, hepatopancreas, cecum, hematopoietic tissue, connective tissue, nervous tissues, haemolymph vessels, stomach and cuticle epithelium, gonads, muscle, antennal gland, heart and appendages (Figure 23).

In the majority of organs, GAV positive signals were mostly detected in the connective tissues. In the hepatopancreas, positive signals were observed in the interstitial connective tissues between the tubules and the organ capsule connective tissues. Hepatopancreas cells were negative for GAV. In the midgut caeca, epithelial cells were negative but spongy connective tissues were positive. The connective tissue of the stomach, directly adjacent to the basement membrane under the epithelial layer also gave positive signals. Few reactions were detected with the cuticular epithelium of stomach, but it did appear that the virus had spread in the epithelium in some foci. Connective cells around the ventral ganglion were also positively detected. In the heart, the connective tissues of the pericard gave a positive signal while myocardial cells were negative. The connective tissues in the antennal gland and between the muscle bundles in the head region gave positive reactions, sometimes very
strong. In haematopoietic tissues, positive cells were detected. Strong positive signals were observed in the central axis and the epithelial pillar cells of the primary and secondary filaments of the gills.

On a cellular level, GAV virus replication was detected in cell cytoplasm but not in the nucleus (Figure 22).

![GAV replication in cell cytoplasm](image.png)

**Figure 22.** GAV replication in cell cytoplasm
<table>
<thead>
<tr>
<th>Lymphoid organ</th>
<th><img src="image1.png" alt="Image" /></th>
<th><img src="image2.png" alt="Image" /></th>
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<tr>
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<td>Connective tissue</td>
<td>Ganglion</td>
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<td><img src="image2" alt="Connective tissue" /></td>
<td><img src="image3" alt="Ganglion" /></td>
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<tr>
<td>Tissue Type</td>
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<tr>
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<tr>
<td>Muscle</td>
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<tr>
<td>Stomach</td>
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<tr>
<td>Appendage</td>
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Figure 23. The target organs of GAV infection in *P. vannamei*. 
4.2.2. Quantification of GAV infection in the lymphoid organ in time

The first signs of GAV infection in *P. vannamei* injected with a dose of $10^3 \text{SID}_{50}$ were observed in the lymphoid organs at 24 hpi (Figure 25). At this time, no positive signals occurred in other organs of infected shrimp. GAV positive signals in gills were only detected in most shrimp at 48 hpi, while results were variable between shrimp for other target tissues and organs.

Because it was consistently positive in all GAV-infected *P. vannamei*, the lymphoid organ was selected as the organ of choice to follow-up the evolution of GAV infection in time.

In the following days, the area of the lymphoid organ positive for GAV signal increased from 1.8% at 48 hpi to 17.1% at 72 hpi (Figure 24). At 120 hpi, the infection reached its greatest expansion, when it occupied about 30% of the lymphoid organ. During the next days until 2 weeks post inoculation, the infected area slowly reduced, still covering up to 22% of the lymphoid organ by the last day (336 hpi).

![Figure 24](image-url)

**Figure 24.** The evolution of GAV infection in the lymphoid organ in time in *P. vannamei* intramuscularly injected with a dose of $10^3 \text{SID}_{50}$. The amount of infection was expressed in percentage of lymphoid organ area positive for GAV signal as detected by IHC.
Figure 25. Time course of GAV infection in the lymphoid organ of *P. vannamei* up to 2 weeks post injection of a $10^3$ SID$_{50}$ dose.
4.3. Characterization of GAV infection patterns in *P. vannamei*

Generally, most GAV-positive signals were observed inside accumulations of virus-positive cells, the so-called spheroids. Within the lymphoid organ, the detected positive signals were markedly more strong in lymphoid organ spheroids (LOS) than in lymphoid organ tubules (LOT) (Figure 26). However, sometimes spheroids within lymphoid organs were observable by the hematoxylin background stain, but were negative for GAV by IHC (Figure 27).

Figure 26. GAV-positive signal was detected both in lymphoid organ spheroids (arrow) and tubules (arrowheads).

Figure 27. GAV-positive (arrow) and negative (arrowhead) lymphoid organ spheroids.
Despite the fact that the most typical appearance of GAV infection we found was in the form of classic, antigen-positive spheroids, positive signals tended to appear in 3 distinctly different patterns in target organs of 3 differently affected shrimp categories (Figure 28).

The first pattern was observed as clusters of infected cells, encapsulated by uninfected fibrous cells. This was categorized as so-called lymphoid organ spheroids or ectopic spheroids (outside lymphoid organs).

The second pattern consisted of signal spread-out through the tissues, without demarcation by fibrous cells. This appearance was often detected in connective tissues, muscles, gills, hematopoietic tissue and the lymphoid organ.

The third pattern looked like unusual “inverted spheroids” in the lymphoid organs. These agglomerations of unstained cells appeared to be encapsulated by fibrous cells which reacted positively with the monoclonal antibody in immunochemistry, but rather faintly compared to normal spheroids.

All three patterns could be found at most time points during the time course study. However, at the later time points of infection, pattern 2 was more represented, and when pattern 3 was found, the shrimp was mostly showing clinical signs.
Figure 28. Three forms of GAV infection in *P. vannamei*.

A, B, E and F: lymphoid organs; C: muscle; D: gills.

### 4.4. Histopathology of GAV infection in the lymphoid organ of *P. vannamei*

Overall, histopathological features of GAV infection in the lymphoid organ were characterized by spheroid formation, pyknosis, karyorrhexis and cellular vacuolization.

The three types of spheroids classified by Hasson et al (1999) were found during our time course study of GAV infection in *P. vannamei*. Type A spheroids are identified as lightly
basophilic and homogeneous cell masses containing no necrotic cells. Type B spheroids are considered as the evolution of type A, but more basophilic and the spheroid bodies contain a number of vacuolated cells as well as pyknotic and karyorrhectic nuclei. Type C spheroids are described as intensely basophilic masses of degenerating cell with numerous vacuoles and also pyknotic and karyorrhectic nuclei.

Although the first positive signals of GAV were detected by immunohistochemistry from 24 hpi, the lymphoid organ still exhibited a normal histological structure of LOT until 48 hpi and absence of LOS (Figure 29). However, by that time, numerous vacuoles as well as aggregations of hemocytes were observed within the inter-tubular connective tissue of the lymphoid organ.

By day 3 after inoculation, the LO of the GAV-infected shrimp contained more histopathological signs of YHD described in previous publications (Figure 30). Abundant basophilic LOS containing hypertrophied nuclei were seen to form between the LOT. Some LOS were surrounded by capsular sheaths. However, the histopathologically typical signs of YHD such as apoptosis and necrosis including nuclear pyknosis, karyorrhexis and cellular vacuolization were seldom observed. It seemed that only type A spheroids occurred at this time and the structures of LOT were normal.

At the next time points (120 and 168 hpi), the LO tissue of infected shrimp showed severe histopathological lesions including numerous pyknotic and karyorrhectic nuclei and vacuolated cells within densely basophilic type B LOS (Figures 31 and 32). The development of LOS could result in distortion and disorganization and loss of normal structure of adjacent LOT. Vacuolization of cells was frequently observed inside LOS which were surrounded by clearly visible, capsular sheaths. No apoptosis or necrosis was seen within LOT.

At 240 and 336 hpi, LOS were highly basophilic, with pyknotic and karyorrhectic nuclei and vacuolated cells (type B and C) (Figure 33).
Figure 29. H&E staining showing the normal histological structures of lymphoid organ tubules (LOT) and absence of lymphoid organ spheroids (LOS) until 48 hpi after GAV inoculation. Numerous vacuoles as well as hemocyte aggregations were observed within the inter-tubular connective tissue of the lymphoid organ during the early stages of infection.
Figure 30. Histopathological features in the lymphoid organs of *P. vannamei* at 72 hpi were characterized by the formation of basophilic spheroids type A surrounded by capsular sheaths.

Figure 31. At 120 hpi, the LO tissue of GAV infected *P. vannamei* showed severe histopathological lesions including numerous pyknotic and karyorrhectic nuclei and vacuolated cells inside densely basophilic type B LOS.
Figure 32. By 168 hpi, numerous pyknotic and karyorrhectic nuclei and vacuolated cells could clearly be observed inside increasingly larger densely basophilic type B LOS.

Figure 33. By 240-336 hpi, the highly basophilic LOS with an abundance of pyknotic and karyorrhectic nuclei and vacuolated cells could also be categorized as type C. The overall aspect of the LO has changed considerably from the normal situation by this time.
4.5. Histopathological features of GAV infection in other target organs

By H&E staining, no histopathological features distinguished the other target organs of GAV-infected shrimp such as gills and hepatopancreas from those of negative control shrimp. Apoptosis, necrosis and dense basophilic inclusions which were reported as cytopathological signs of YHD (Khanobdee et al, 2002) were infrequently detected in connective tissue of hepatopancreas and other organs. Although pyknotic and karyorrhectic nuclei were randomly scattered in gills and other organs, the information was not enough to confirm whether their existence was due to the GAV infection.

<table>
<thead>
<tr>
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<th>Uninfected tissue</th>
<th>Infected tissue</th>
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<tr>
<td><strong>Gills</strong></td>
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<tr>
<td><strong>Hepatopancreas</strong></td>
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Figure 34. Signs of apoptosis with pyknotic (arrows) and karyorrhectic nuclei (arrowheads) in GAV-infected tissues of gills and hepatopancreas (right) in comparison with the same structures of uninfected tissues (left).
Clinical signs of GAV infection in *P. vannamei*

In our study, subadult SPF *P. vannamei* experimentally challenged with a dose $10^3 \text{SID}_{50}$ did not show typical clinical signs of YHD with yellowish discoloration of the shrimp cephalothorax (YHD) as reported in Thailand (Chantanachookin et al. 1993) or signs of reddish coloration of GAV infected shrimp as reported in Australia (Spann et al, 1997). The few abnormalities which were seen included: anorexia, lethargy, soft shell, and pale coloration in 10% of inoculated shrimp. These gross signs of GAV infection were also reported in previous publications in *P. monodon* by Chantanachookin et al. (1993), Spann et al. (1995, 1997, 2000 and 2003) and in *P. vannamei* by Lu et al (1995), Anantasomboon et al (2008) Tang et al. (2002).

Thus, the remarkable sign of yellow head discoloration was likely to appear in the natural host *P. monodon* infected by YHV-type 1. However, up to now, the mechanism of yellowish development caused by YHV type 1 infection in *P. monodon* has remained unknown.

Second, no mortality was recorded in *P. vannamei* during the 2 weeks GAV challenge experiments.

Both these findings differed considerably from what has already been published on YHCV infection and disease in *P. vannamei*. Tang et al (2002) informed that GAV could cause disease and mortality in infected *P. vannamei* (1-2 g) with survival rate of 33% on the 14th day post injection. In other experiments challenging with YHV type 1, infected *P. vannamei* (7-10 g) showed only 14% survival rate after 10 days (Anantasomboon et al., 2008). Possibly, the *P. vannamei* shrimp in our study had a lower susceptibility to GAV disease thanks to their older age and larger size. In any case, our *P. vannamei* suffered dramatically less disease due to GAV infection than *P. monodon* (Spann et al. 1997, 2000 and 2003). This could indicate that *P. vannamei* is genetically less susceptible to disease due to GAV infection than *P. monodon*.

The results in our study also reconfirmed that the virulence of GAV/YHV type 2 is considerably lower than that of YHV type 1. When challenged with YHV type 1, 100% mortalities of *P. vannamei* were noticed 5 days after injection (Anantasomboon et al, 2008), even when the dose was diluted up to $10^{-10}$ (Sittidilokratna et al, 2009).
Target organs of GAV infection in *P. vannamei*

IHC staining showed GAV infection in *P. vannamei* in nearly all major organs, predominantly in connective tissues. The results were similar to those previously reported by Chantanachookin et al. 1993, Lu et al 1995, Spann et al., 1997, Munro et al, 2007.

The cuticular epithelium was also a target organ of GAV replication in the study. Likewise, Tang et al (2002) observed a strong reaction for GAV by ISH in the cuticular epithelium of the stomach in experimentally infected *P. vannamei*. According to Chantanachookin et al (1993), Spann et al., 1997 and Tang et al., 2002, hemocytes also support GAV replication. In the present study, we were only able to check for GAV signals by indirect immunofluorescence staining of hemocytes from a very limited number GAV-infected shrimp due to a lack of experimental animals (data not shown). As a result, the obtained data was insufficient to make a solid conclusion.

In the present study, the organ distribution of GAV varied quite strongly from individual to individual and during the course of infection. However, 100% of shrimp inoculated with a GAV dose of $10^3$ SID$_{50}$ showed clearly visible positive signals on IHC in the lymphoid organ from 24 hpi, while all other organs were still negative at that time. These findings showed that the lymphoid organ in *P. vannamei* is a much more prominent and consistently infected target organ of GAV than the gills, in which the presence of severe GAV infection was the origin for the name (Spann et al., 1997). It had already been mentioned by many scientists (Chantanachookin et al., 1993, Lu et al, 1995; Spann et al., 1997; Tang et al, 1999 and 2002; Wang et al, 2000; Cowley et al, 2001; Kanobdee et al, 2002; Soowannayan et al, 2002; Munro et al, 2007; Rusaini et al, 2010; Duangsuwan et al, 2008 and 2011) that the lymphoid organ plays an essential role as a primary site of GAV replication in shrimp, but we would like to go further. Seeing the relatively sparse infection in the gills of *P. vannamei*, we suggest to refrain from calling the virus GAV, but rather use the official ICTV genus name: Okavirus. In this context, “Oka” refers to the Japanese name for the LO. Alternatively, the name “lymphoid organ virus” mentioned by Spann (1995) would also be a suitable name for the virus.

During the time course study, the tissue distribution of GAV seemed to vary according to the phase of infection. It was previously suggested that shrimp are able to control and maintain chronic asymptomatic GAV infections by the process involving the lymphoid organ spheroids (Spann et al., 2003; Anantasomboon et al., 2008). As a result of that, GAV
infection was only detected in LOS rather than in other organs in healthy cultured *P. monodon* in Australia. Conversely, acute phase infections and occurrence of disease seemed to linked with the systemic distribution of virus in connective tissues throughout the cephalothorax (Spann et al, 2003; Duangsuwan et al, 2008, Rusaini et al, 2010). This phenomenon does not seem to occur in *P. vannamei*, as even shrimp with wide-spread infection remain clinically healthy.

In any case, further research is needed to map in more detail the exact localization of GAV infection in *P. vannamei* shrimp, as well as the role of hemocytes in the GAV infection.

**Quantification of GAV infection in the lymphoid organ in time**

During the course of the 2-week experiment, the amount of positive signal in the lymphoid organ increased to reach a peak at 5 days, after which the amount slowly decreased. This signal was present mostly inside LOS, which were also visible in the histopathological observations in the study. There is however a possibility that the positive signals observed at later time points are due to inactivated viral particles remaining in LOS as residues from shrimp defense reactions.

Indeed, according to Anggraeni et al. (2000), Khanobdee et al. (2002), Anantasomboon et al. (2008), Duangsuwan et al. (2008) and Rusaini et al. (2010), the reduction of GAV infection in the lymphoid organ and the formation of spheroids are related to the shrimp immune response to the viral pathogen. The viral components would be transferred by the phagocytic hemocytes into the spheroid bodies in which viral quarantining and clearance were conducted (Hasson et al, 1999; Anggraeni et al, 2000, Duangsuwan et al, 2008, Rusaini et al, 2010). Consequently, once under immune control, GAV infection was reduced in challenged shrimp and positive signals were only detected within LOS. Further investigations are still needed to fully confirm this.

**Characterization of GAV infection patterns in *P. vannamei***

Infected cells contained GAV antigen in their cytoplasm.
Within the lymphoid organ, the detected positive signals were significantly stronger in LOS than in LOT. This was in accordance with the reports of Soowannayan et al. (2003), Spann et al 2003 and Anantasommoon et al (2008) who used IHC or in situ hybridization (ISH). However, not all of the observable spheroids within lymphoid organs found in our study had positive reaction with immunohistochemistry. The reason for this remained unclear.

By histopathology and IHC, three scenarios of GAV infection were determined in *P. vannamei*. First, GAV infection was only found in LOS and few ectopic spheroids were detected in other target organs such as gills, appendages and connective tissues at other times. The spheroid bodies or ectopia were sequestered by visibly surrounding fibrous cells. The viral infection seemed to be contained or under immune control.

In the second pattern of GAV infection, spheroids were absent except for in the lymphoid organs. The infection was mostly found spread throughout muscles and connective tissues form, but not in ectopic spheroids.

The third presentation was characterized by a great amount of positive signals in many organs. Spheroids and ectopic spheroids were detected in most target organs. In the lymphoid organ, the GAV positive signals were found both in LOS and in LOT. Remarkable was the occurrence of peculiar “inverted spheroids”, in which clusters of unstained cells were surrounded by cells with positive signal for GAV. The latter scenario was considered as an infection “out of immune control”, in which a severe infection occurred in all major organs.

The first and second types of infection appeared in GAV-infected shrimp without clinical signs whereas the third was related to infected shrimp with observable abnormalities such as anorexia, lethargy and pale coloration.

The precise mechanism behind the formation of different GAV infection patterns is unknown. According to Anantansoomboon et al (2008) and Duangsuwan et al (2008), the formation of spheroids appeared to be related to shrimp tolerance to virus infection or to chronic stages of infection. This could be a possible explanation for the first and second presentations but not the third one. The severity of GAV infection in the third scenario showed that the immune control of infected was lacking and that this resulted in the disruption of the physiological processes in the infected shrimp, leading to disease. It is possible that, as a result of that, death or slow growth could follow in the following weeks.
From field observations, it is known that GAV is highly prevalent (up to 100% in some areas) in healthy farmed *P. monodon* in Australia (Oanh et al., 2011) and that the infection with the virus is likely associated with decreased production (Munro et al., 2011). Further studies elaborating on our study are needed to find out whether GAV infections in *P. vannamei* do or do not pose a similar threat.

**Spheroid formation in the lymphoid organ and relation to chronic GAV infection**

The lymphoid organ of penaeid shrimp was identified to function as a haemolymph filter and play a critical role in the immune defence of penaeid shrimp against pathogens. It has already been shown that the lymphoid organ was a major target organ of viral infection and that it is probably involved in viral elimination (Hasson et al., 1999; Anggraeni et al., 2000; Duangsuwan et al., 2008; Rusaini et al., 2010). Particularly the formation of spheroids within the lymphoid organ was related to chronic viral diseases in general, and YHD in particular (Duangsuwan et al., 2008; Anantasombom et al., 2008), but their exact formation and function remain unknown.

Histopathological changes in the lymphoid organs of GAV-infected shrimp were demonstrated by Spann et al. (1997, 2003) and Duangsuwan et al. (2008) in *P. monodon* and by Anantasombom et al. (2008) in *P. vannamei*.

The present study also presented histopathological examinations of GAV infection in *P. vannamei*, focusing on the lymphoid organ. We mainly noted spheroid formation, pyknotic and karyorrhectic nuclei and vacuolated cells from 72 hpi. These signs indicate an ongoing process of necrosis or apoptosis.

For YHV type 1 infection, histological abnormalities were reported to appear earlier at approximately 36 hpi in infected *P. monodon* (Khanobdee et al., 2002) and at 48 hpi in infected *P. vannamei* (Anantasomboon et al., 2008). By TEM, virus particles were even found in lymphoid cells as early as 18 hpi (Duangsuwan et al., 2011). Hence, Chantanachookin et al (1993) suggested that histopathology should be only used for assistant diagnosis of GAV infection in shrimp at late stages. More specific detection methods such as IHC, in situ hybridization (ISH) or PCR should be applied, especially for diagnosis at earlier stages of GAV infection.

Three types of LOS (A, B and C, according to Hasson et al (1999)) and their transition were detected during the course of GAV infection in *P. vannamei*. LOS forms and transformations
were believed to play roles in quarantining and clearance viruses (Duangsuwan et al, 2008) and related to chronic infection of GAV (Spann et al, 2003). These suggestions were supported by results from IHC and ISH performed Soowannayan et al. (2003), Spann et al. (2003) and Anantasomnoon et al. (2008). These authors also emphasized that, in chronic infection, the localization of the virus was only detected in the LOS while in acute infection mostly detection was in LOT. To explain this phenomenon, it was hypothesized that, in the earlier phase of viral infection, viral components would be in the stromal matrix cells and be gradually transferred by the phagocytic hemocytes into the spheroid bodies, in the later or chronic phase of the infection (Hasson et al, 1999; Anggraeni et al, 2000, Rusaini et al, 2010). In acute infection, the morphological evolution of LOS from type A to type C represented the viral sequestering stages during which the virus was quarantined and eliminated by apoptosis and necrosis. But, in chronic infection, a certain amount of viral particles still remained, thus the LOS formation was persistent (Duangsuwan et al, 2008). This processes could result in the abundance of LOS in the lymphoid organ of GAV infected shrimp after 2 weeks, as was also observed in the present study.

Also, about the role of LOS in YHD, another hypothesis was that spheroid formation was associated to the tolerance of *P. vannamei* to YHV type 1 (Anantasomnoon et al., 2008). The survivors from acute infection were discovered to be tolerant rather than resistant to YHV type 1. This process appeared to be related to low viral loads and lymphoid organ spheroid formation. It was suggested that the shrimp responded to the challenge virus by rapidly passing from the early stage of YHV infection (with clearly histopathological lesions in the LOT) into the persistently infected carrier state with normal LOT and the forming of LOS. During this time, only LOS gave positive signals of YHV infection. However questions as to whether YHV type 1 tolerance could result from innate genetic tolerance or adaptive tolerance or both remain unanswered.

In contrast to the situation in *P. monodon* described above, and our results, Tang et al (2002) stated that spheroids were absent in the lymphoid organ of GAV-infected *P. vannamei*, although positive signals were found in LOT by in situ hybridization (ISH).

**Apoptosis and GAV infection**

Other typical histopathological lesions related to GAV infection in the *P. vannamei* in our study included cellular necrosis and apoptosis.
During our study, pyknosis, karyorrhexis and vacuolated cells were not observed in LOT but clearly visible from the day 5th post injection onwards in LOS type B and C. These cellular changes could also been seen in modest numbers in gills and the inter-tubular connective tissues of hepatopancreas. The results of the study were different from those of previous publications. Anantasomboon et al (2008) reported the common appearance of pyknotic and karyorrhecti nuclei in the LOTs, already from the second day post injection of YHV-type 1 in *P. vannamei*.

Several publications on YHD in shrimp have reported the occurrence of apoptosis (Chantanachookin et al., 1993 & Khanobdee et al., 2002; Khanobdee et al., 2002; Anantasomboon et al., 2008). Due to absence of inflammatory reactions, Khanobdee et al. (2002) suggested that cell death in YHD resulted mainly from apoptosis. Hence, high numbers of apoptosis cells could result in the death of YHV-infected shrimp because of the ensuing function loss of vital organs. However, histopathological observation of pyknotic and karyorrhecti nuclei and vacuolated cells alone does not allow for a definite distinction between apoptosis - programmed cell death- and necrosis. In our opinion, more specific stainings are needed to investigate this.

Compatible with the evidence that apoptosis plays and important role in YHV infections in shrimp (Khanobdee et al., 2002), the viral accommodation theory (Flegel et al, 1998 and Flegel, 2001) proposed that apoptosis was the cause of shrimp death from viral infections. According to this theory, the high prevalence of GAV infections in healthy shrimp in Australia and the apparent tolerance observed in shrimp surviving YHV infections could be consequences from two assumptions. On the one hand, the shrimp or their pathogens could have genes that inhibited apoptosis. On the other hand, the host-virus interaction resulted in specific memory that suppressed apoptosis. In both cases the shrimp could avoid disease or death but would still carry virus infection. These theories could be possible explanations why were observed 100% survival in GAV-infected *P. vannamei* in the present study. Hence, further understanding of apoptosis can lead to interesting insights for management and therapeutics for shrimp viral disease in general and of YHD in particular.
CHAPTER 6 - CONCLUSIONS

1. Inoculation of GAV (YHV type 2) in *P. vannamei* by intramuscular injection did not result in typical YHD symptoms. Some abnormalities such as anorexia, soft shell and pale color could be observed, but this was rare (10% of shrimp) and often transient. Hence it was concluded that the GAV infections occurred mainly subclinically.

2. GAV infections did not result in any mortality of *P. vannamei*.

3. On IHC analysis, the GAV infection in *P. vannamei* was systemic. GAV-positive signals were detected in the cytoplasm of infected cells in most major organs of infected shrimp. The lymphoid organ was found to be the target organ of choice for studying GAV infections in *P. vannamei*.

4. The earliest time point at which GAV infection could be detected was 24 hpi. This was in lymphoid organs. GAV replication was slow to become detectable in other organs (by 48 hpi). The progression of the infection in the lymphoid organ rose dramatically from only 1-2% infected area at 48 hpi to 17% at 72 hpi. The biggest area of GAV positive signal was observed at 120 hpi with 30%. After that, infected areas gradually reduced to 27%, 25% and 22% at time points of 168, 240 and 336 hpi, respectively.

5. Histopathological examination of GAV-infected *P. vannamei* showed the presence of typical lymphoid organ spheroids from 72 until 336 hpi. Apart from that, little or no pathology was seen in the rest of the body.
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