

Yellow head virus outbreaks in intensive freshwater culture of Pacific white shrimp (*Litopenaeus vannamei*) in Thailand and its experimental infection at different salinity levels

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ABSTRACT

Litopenaeus vannamei (5-10 g) with gross signs of YHV disease (faded overall body, yellowish cephalothorax and high mortality) were collected in 15 farms located in Ratchaburi province, central Thailand during June 2009 to January 2010 and analysed for YHV infection using RT-PCR. Results revealed YHV infection in those coming from 12 of the 15 farms, while the samples from the remaining 3 farms gave negative results for YHV infection. Analysis of RT-PCR amplicons from infected shrimp revealed the presence of YHV type 1-b (alone). Histopathological study of infected shrimp showed severe necrosis with prominent nuclear pyknosis and karyorrhexis in the gills, lymphoid organ, hepatopancreas and walls of the stomach. The immunoreactivity with YHV p20 nucleocapsid protein was strong in the tissues of the said body parts. Experimental injection and feeding at different salinity levels were performed to determine the severity of YHV infection in *L. vannamei*. Three hundred normal (Specific Pathogen Free) white shrimp (8-10g) were transported from the source farm to the Aquaculture Business Research Center laboratory at Kasetsart University and acclimated at the salinity of 5, 15 and 30 practical salinity unit (psu) (100 shrimp for each treatment) for 10 days before the experiment. Thirty shrimp from each salinity were infected by intramuscular injection at the fifth abdominal segment with the extracted filtrate from gills and swimming legs of YHV-infected shrimp, through 450 nm at 0.1 ml. Infected shrimp from each salinity were placed in three aquaria with 10 shrimp per aquarium. In the second experiment, YHV infected shrimp were cut into several pieces and fed to 90 normal shrimp for one meal (30 shrimp from each salinity) before placing into aquaria (10 shrimp per aquaria). Mortality was observed throughout the 40 days experimental period. Cumulative mortality was also recorded and compared among the treatment groups. Some moribund shrimp were preserved in 80% ethanol for RT-PCR assay. Results showed that moribund shrimp developed yellowish coloration of the cephalothorax and gills. The first mortality was

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observed within 24 hours, followed by severe mortality until day 6. This result indicated that mortality in low salinity water (5 psu) was significantly faster than those at 15 and 30 psu groups. At 30 psu, the injected shrimp could survive more than 20 days after injection. Results from the feeding experiment demonstrated that the first mortality was observed after 48 hours. Similar results were observed in which mortality in the low salinity group (5 psu) was faster than those at 15 and 30 psu groups. At 30 psu, the first shrimp died after 72 hours and one shrimp survived until day 40. RT-PCR assay confirmed that dead shrimp from the two experiments were severely infected by YHV. However, slight infection was observed in the only surviving shrimp.

Keywords : Yellow head virus, *Litopenaeus vannamei*

INTRODUCTION

Yellow head virus (YHV) has caused mass mortality in farmed black tiger shrimp (*Penaeus monodon*) in Thailand since 1990 (Limsuwan, 1991). The virus was named from the gross signs of disease which included a yellowish cephalothorax and very pale overall coloration of moribund, infected shrimp. The causative agent is a bacilliform, enveloped, (+) ssRNA virus classified in the genus Okavirus, family Roniviridae, Order Nidovirales (Boonyaratpalin *et al.*, 1993; Chantanachookin *et al.*, 1993, Walker *et al.*, 2005). Histologically, YHV infections can be easily recognized by densely basophilic inclusions, particularly in H&E stained gill sections and rapidly stained whole gills (Flegel, 2006). Severe necrosis of the lymphoid organ (LO) is one of the most conspicuous and “typical” lesions caused by YHV. Shrimp with acute YHV infection displays a generalized multifocal to diffused severe necrosis, with prominent nuclear pyknosis and karyorrhexis, accompanied by extensive degenerative changes of this organ (Boonyaratpalin *et al.*, 1993; Chantanachookin *et al.*, 1993; Lu *et al.*, 1994; Wang *et al.*, 1996)

YHV has been reported widely in Asia, while other genotypes in the yellow head disease complex (including gill associated virus, GAV) have been reported in healthy *P. monodon* from Australia, Asia and Africa (Wijegoonawardane *et al.*, 2008c). A recent study revealed that six geographical types of YHV have been described (YHV-1 to YHV-6). These types differ in virulence and in overall genome sequences by up to 20% (Cowley *et al.*, 1999; Sittidilokratna *et al.*, 2002; Wijegoonawardane *et al.*, 2008a). The presence of different YHV genotypes in various countries in Indo-Pacific areas has also been reported but the most virulent type (YHV Type-1) was reported only from Thailand (Wijegoonawardane *et al.*, 2008b). Additionally, genotype YHV-1 was further subdivided into YHV-1a and YHV-1b based on a 54-amino acid deletion in the portion of encoding structural protein gp 116 in YHV-1b when compared to YHV-1a (Sittidilokratana *et al.*, 2009). Detailed diagnostic protocols utilized to investigate for presence of viruses within the yellow head disease complex, including the use of histopathology, immune-histochemical techniques and nucleic acid detection are provided by the OIE (2006).

Domesticated and genetically selected, specific pathogen-free (SPF) *L. vannamei* has replaced *P. monodon* as the dominant cultivated shrimp species in Thailand since 2002 (Wyban, 2007a, b). In 2006, YHV outbreaks were found in *L. vannamei* cultured only in freshwater areas in Thailand (Senapin *et al.*, 2010). In contrast, YHV outbreaks in *P. monodon* were found in both freshwater and coastal areas. There is no report about the prevalence of YHV types that may have caused the continuing outbreaks in Thailand or whether salinity levels affect the mortality of the infected shrimp. The objective of this studies was to determine the type of YHV causing the outbreaks in *L. vannamei* farms located in freshwater areas in Ratchaburi province, central Thailand during June 2009 to January 2010. The severity of YHV infection in *L. vannamei* by experimental injection and feeding at different salinity levels was also studied.

MATERIALS AND METHODS

The determination of YHV type

Shrimp samples

L. vannamei (5-10 g.) with gross signs of YHV disease (faded overall body, yellowish cephalothorax and high mortality) were collected from 15 farms located in Ratchaburi province, central Thailand during June 2009 to January 2010.

RNA extraction and histopatholgical examination

Gill tissues from individual shrimp were homogenized in Trizol reagent

(Invitrogen), and RNA was extracted following manufacturer's instructions. RNA concentration and quality were measured by spectrophotometric analysis at 260 and 280 nm. Shrimp samples were preserved in Davison's fixative for 24 hours and then transferred to 70% ethanol solution prior to processing as previously described by Bell and Lightner (1988). All samples were sectioned and stained with hematoxylin and eosin (H & E).

YHV typing and nested PCR

YHV Primers were designed according to Wijegoonaawardane *et al.* (2008c) and nested RT-PCR was performed according to Senapin *et al.* (2010) to determine whether the samples were YHV-1a or YHV-1b. The expected nested RT-PCR result of YHV-1a is 555bp while, the product of YHV-1b is 393 bp.

Immunohistochemical detection of YHV

Shrimp dissection, fixation and paraffin-embedding procedures were based on Bell and Lightner (1998). The tissues were sectioned at 5-7 μ m thickness and placed on positively charged microslides. Immunolabeling and immunohistochemistry were carried out according to Sithigorngul *et al.* (2007)

The severity of YHV infection in *L. vannamei* at different salinity levels

Shrimp samples

Three hundred Specific Pathogen Free (SPF) *L. vannamei* (8-10 g) were transported from the source farm to the Aquaculture Business Research Center laboratory at Kasetart University and acclimated in three

500-L fiberglass tanks with aeration at 5, 15 and 30 practical salinity unit (psu). A hundred shrimp were kept for each salinity for 10 days before the experiment. During this period, shrimp were fed four times daily with commercial pelleted feed for *L. vannamei*.

YHV infection

Thirty shrimp from each salinity were infected by intramuscular injection at the fifth abdominal segment with the extracted filtrate from gills and swimming legs of YHV-infected shrimp from experiment 1, through (450 nm) filter at 0.1 ml per shrimp. Infected shrimp from each salinity was placed in three (150 liter) aquaria with 10 shrimp per aquarium. In the second experiment, YHV infected shrimp were cut into several pieces and fed to 90 normal shrimp for one meal. (30 shrimp from each salinity) before placing into (150 liter) aquaria. Shrimp were fed four times daily with commercial pelleted feed for *L. vannamei*.

YHV detection using RT-PCR

IQ2000 YHV/GAV RT-PCR detection kit was used for YHV detection and RT-PCR was performed following manufacturer's instructions. Amplified products were analysed on 2% agarose gels.

Water quality control

Water quality parameters such as pH, dissolved oxygen (DO), ammonia and nitrite were monitored throughout the experiment to ensure that all water quality parameters were suitable for *L. vannamei* cultured as described by Limsuwan and Chanratchakool (2004).

Statistical analysis

Mortality was observed throughout the 40 days experimental period. Cumulative mortality were recorded and compared among the treatment groups using one-way analysis of variance followed by Duncan's multiple range test.

RESULTS AND DISCUSSION

The determination of YHV type

The nested RT-PCR result revealed the expected amplicon of 393 bp from all samples of *L. vannamei* collected from the 12 studied farms, while the other samples from the 3 studied farms gave negative results for YHV infection. The RT-PCR product clearly indicated that this YHV outbreak was caused by YHV-1b alone (Fig. 1). Affected shrimp had pale body colour, with mortality starting from 40-60 days of culture, with a cumulative mortality of 40-80%. Histological examination of YHV-1b infected shrimp revealed severe necrosis, with prominent nuclear pyknosis and karyorrhexis in the hemocytes, gills, lymphoid organ stromal matrix cells and some other tissues, including the connective tissue and spongy connective tissue and walls of the stomach (Fig. 2) similar to the report of Flegel (2006). Meanwhile, the immunoreactivity with YHV p20 nucleocapsid protein was strong in gill tissues, hepatopancreas and walls of the stomach (Fig. 3). This result is similar to the study by Senapin *et al.* (2010) wherein they reported an infection of YHV-1b *L. vannamei* in 4 ponds in Ratchaburi province and 3 ponds in Nakhon Pathom

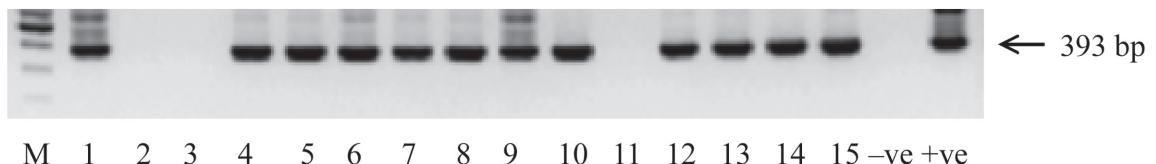


Figure 1. Agarose gel showing RT-PCR products of YHV-1b (diseased shrimp) from 15 studied farms. M=DNA Marker, +ve=positive control (YHV-1b), -ve=negative control (SPF shrimp)

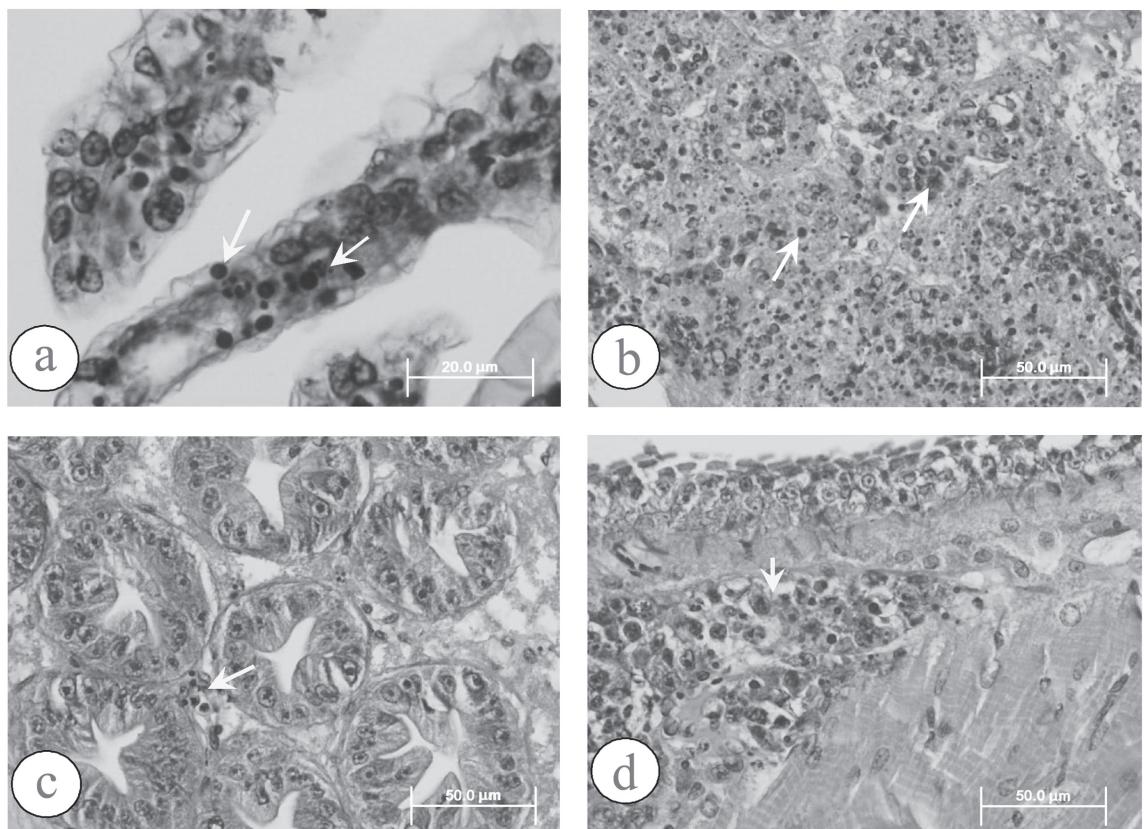


Figure 2. Microscopic observation of
 (a) gills (H&E, bar=20 μ m),
 (b) lymphoid organ (H&E, bar=50 μ m),
 (c) hepatopancreas (H&E, bar=50 μ m),
 (d) wall stomach (H&E, bar=50 μ m),
 show severe necrosis with prominent nuclear pyknosis and karyorrhexis of *L. vannamei* infected with YHV

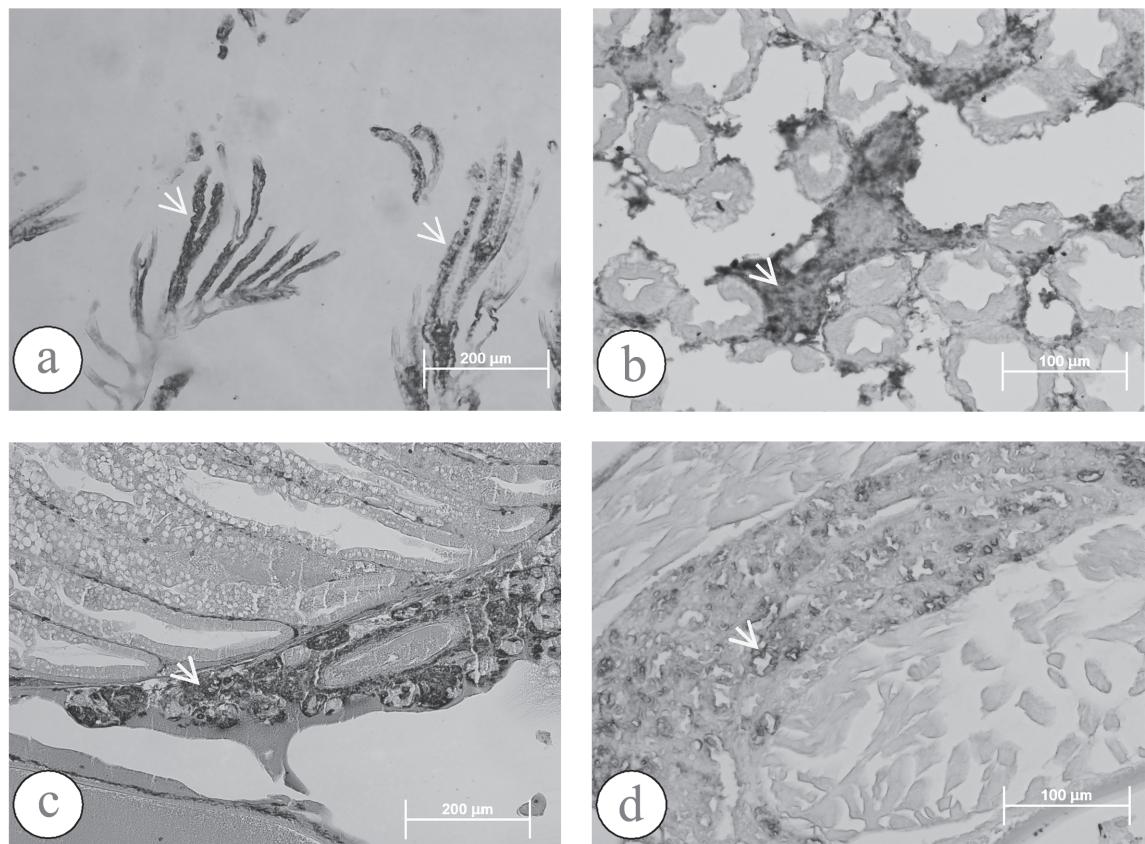


Figure 3. Immunohistochemical localization of YHV infection in tissues of *L. vannamei* showing strong immunolactivity of YHV p20 nucleo capsid protein (arrows).
 (a) gills (bar=200 μ m),
 (b) hepatopancreas (bar=100 μ m),
 (c) lymphoid organ
 (d) hemopoietic tissue (bar=100 μ m)

province, Thailand, during December 2007 to March 2008. Sittidilokratna *et al.* (2009) analysed proteins from purified virions from a previous YHV-1b isolate from Ratchaburi province in 2006 and showed the absence of full-length gp116 together with the deletion version, suggesting that it was also an infection by YHV-1b alone.

A study by Wijegoonawardane *et al.* (2008b) reported that YHV type-1a infection

was found in 18 samples of *P. monodon* collected in Thailand from 2000 to 2003. In contrast, YHV type1-b infection found in *L. vannamei* from freshwater cultivation areas was first recorded in 2003 after *L. vannamei* became the dominant cultured species in Thailand. It is possible that YHV-1b may have originated as a mutant of YHV-1a and might be more suitable for the infection of *L. vannamei* than type-1a (Senapin *et al.*, 2010).

The severity of YHV infection in *L. vannamei* at different salinity levels

Results from the experimental injection method are shown in Fig. 4. The first mortality was observed within 24 hours, followed by severe mortality until day 3, with all shrimp from the three groups dead

at day 20. However, mortality in the low salinity water (5 psu) was significantly faster than those at 15 and 30 psu groups. At 30 psu, the injected shrimp survived more than 20 days after injection.

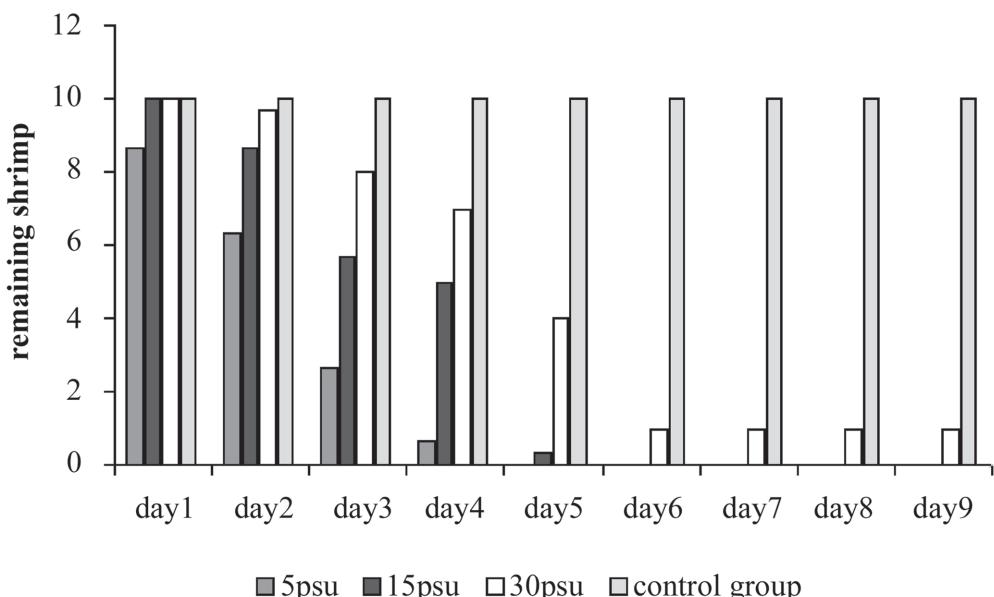


Figure 4. Number of surviving shrimp after being injected with YHV

Results from the feeding experiment are shown in Fig. 5. The first mortality was observed after 48 hours. Similar results were observed wherein mortality in the low salinity group (5 psu) was faster than those at 15 and 30 psu groups. At 30 psu, the first shrimp died after 72 hours and one shrimp remained alive at day 40. RT-PCR assay confirmed that the dead shrimp from the two experiments were severely infected by

YHV (Fig. 6). However, light infection was observed in the one shrimp which was alive at the end of the experiment. This indicated that a high salinity level (30 psu) could reduce the mortality rate of the experimental shrimp. This is mainly because a salinity level of 30 psu is suitable for the natural life cycle of *L. vannamei* as described by Boone (1931). Stern *et al.* (1990) reported that *L. vannamei* can tolerate a wide range of

salinities, from slightly brackish (1–2 psu) to hypersaline (40 psu). However, the optimum salinity for shrimp for ecophysiological performance is 25–30 psu (Bray *et al.*, 1994). A previous study comparing YHV challenge results in *P. monodon* with those for various palaemonid shrimp indicated that tolerant palaemonid species carried YHV as persistent infections characterized by what appeared to be specific suppression of one of the YHV envelope proteins gp116 when compared with the capsid protein p20 (Longyant *et al.*, 2005). Anantasomboon *et al.* (2008) indicated that the ability of these shrimp to tolerate viral infections might be related to the control over viral envelope protein production by an unknown mechanism. Presumably, the lack of one envelope protein (gp116) would reduce the ability of the virus (YHV-1b) to spread within the host.

Because all the studied farms were stocked with Specific Pathogen Free (SPF) postlarvae, the horizontal transmission from infected shrimp or natural YHV carriers through the water and by cannibalism of weak or moribund shrimp may be the major source of YHV infection. Flegel *et al.* (1995a, b; 1997) reported that YHV remains viable outside the host, in aerated seawater, for up to 72 hours and the agent can be inactivated by heating to 60°C for 15 min and by exposure to 30 ppm chlorine. Several studies have reported that YHV can be reproduced by direct injection or ingestion of infected tissue or tissue extracts and by co-habitation of normal shrimp with infected carriers such as *Penaeus aztecus*, *P. duorarum*, *P. japonicas*, *P. monodon*, *P. setiferus*, *P. stylirostris*, *Metapenaeus brevicornis*, *M. affinis*, *Palaemon styliferus* and *Macrobrachium lanchesteri* (Lightner

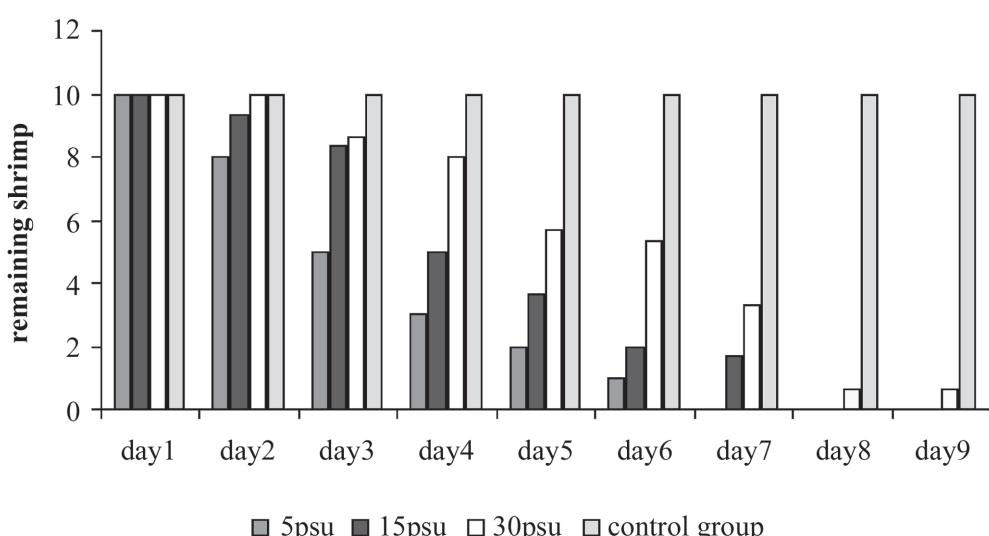


Figure 5. Number of surviving shrimp after feeding with YHV-infected shrimp

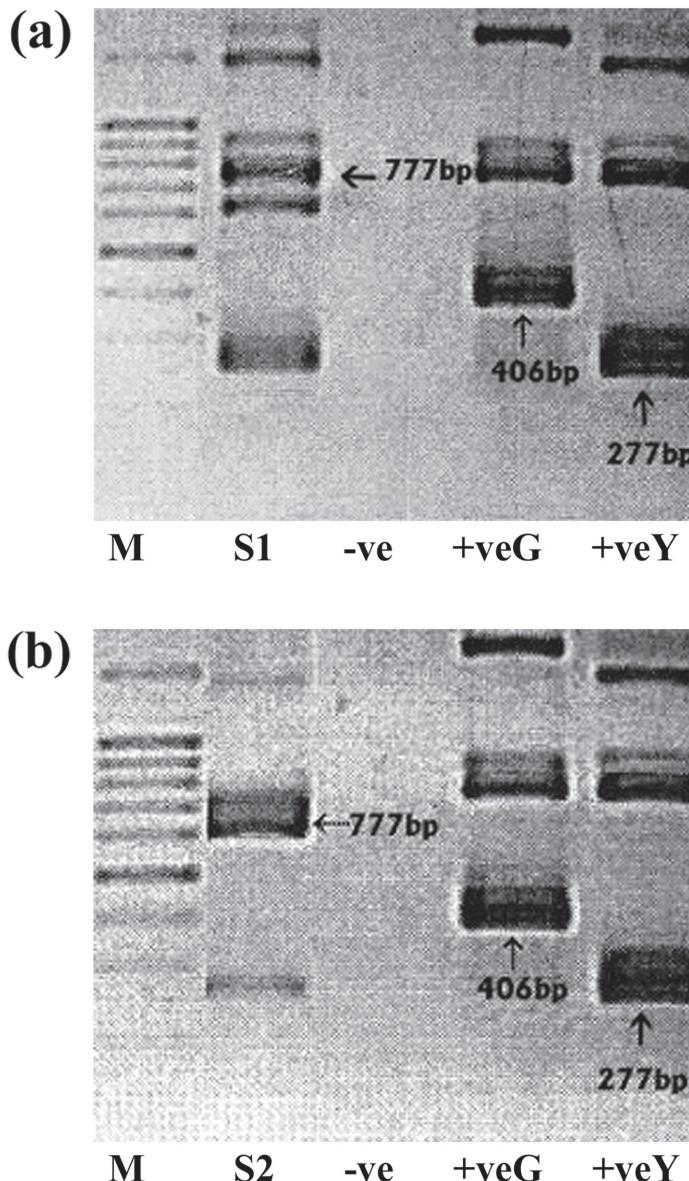


Figure 6. Diagnostic results of YHV-1b infected samples using a commercial RT-PCR detection kit.

a) shrimp injected with YHV-1b;
 b) shrimp fed with YHV1-b infected shrimp.

Bands at 277 and/or 777 bp indicate YHV infection according to kit instruction.
 M=DNA Marker;
 S1 - shrimp injected with YHV-1b;
 S2-shrimp fed with YHV1-b infected shrimp;
 +ve G-positive control (GAV);
 +ve Y-positive control (YHV);
 -ve-negative control (SPF shrimp)

and Redman, 1998; Longyant *et al.*, 2006; Stentiford *et al.*, 2009). The best way to prevent YHV disease is to eliminate potential YHV carriers from the culture system by proper pond preparation and by subsequent filtration of exchange water (Senapin *et al.*, 2010). In places where there is a high risk of viral infection, farmers should disinfect the water with calcium hypochlorite before stocking the postlarvae, and should buy postlarvae only from breeders who use SPF broodstock. The farmers should have a reservoir pond and use only disinfected water which has been left to settle for at least 2 weeks when changing the water in their grow-out ponds.

CONCLUSIONS

YHV outbreak in this study was caused by YHV-1b alone. Keeping *L. vannamei* at high salinity (30 psu) could delay clinical signs and onset of mortalities when infected with YHV-1b.

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