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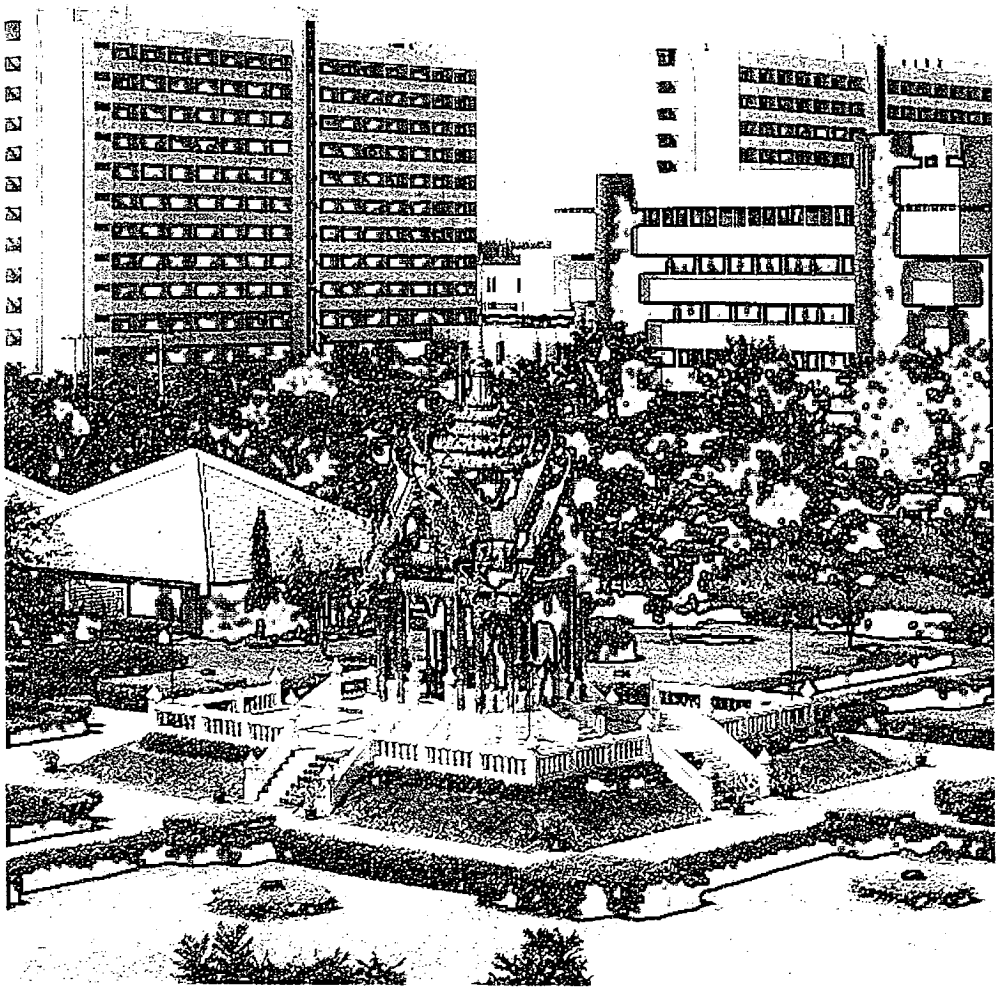

DIVISION OF MEDICINE AND HEALTH SCIENCES

RANGSIT UNIVERSITY

(Thailand)

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Re-infection Study of the Monodon Slow Growth Agent in Black Tiger Shrimp *Penaeus monodon* and the Presence of Similar Viral Particles in other Commercial Shrimp Species

Gun ANANTASOMBOON[1*], Kanokporn CHAYABURAKUL[1], Waraporn SAKEAW[2,3] Anutara BOON-NAT[4] and Boonsirm WITHYACHUMNARNKUL[2,3]

Anatomy Unit[1], Department of Medical Science, Faculty of Science, Rangsit University, Muang Ake, Pathumthani 12000, Thailand*

Center of Excellent for Shrimp Molecular Biology and Biotechnology (Centex Shrimp)[2], and Department of Anatomy[3], Faculty of Science, Mahidol University, Bangkok 10400, Thailand

Shrimp Cultural Research Center[4], Charoen Pokphand Foods (CPF) Public Company, Samutsakorn 74000, Thailand

ABSTRACT

To determine the cause of monodon slow growth syndrome (MSGS) in the black tiger shrimp *Penaeus monodon*, re-infection experiment was carried out by injection of bacterial free lymphoid organ extracts (LOE) from two commercial shrimp species into healthy juvenile *P. monodon*. The shrimps of which LOE were obtained, were MSGS *P. monodon*, specific pathogen-free (SPF) white Pacific shrimp *P. vannamei* that were co-cultured with MSGS *P. monodon*, SPF *P. vannamei* that were mono-cultured, and *P. monodon* that had normal growth rate. Both groups of the *P. vannamei*, from which LOE were taken, did not have growth retardation. Four and a half months post-injection, *P. monodon* groups that received LOE from MSGS *P. monodon* and *P. vannamei* that were co-cultured with MSGS *P. monodon* had clinical manifestations of MSGS, i.e., growth retardation, size difference and increased body color. Under light microscopy (LM), the lymphoid organ of the naturally occurred MSGS *P. monodon* contained several spheroids with inclusion bodies of different size and colors in the spheroidal cells and similar features were observed in the gills. Under transmission electron microscopy (TEM), numerous icosahedral virus-like particles at 25 nm in diameter, and without envelope, were observed in association with the inclusion bodies. Besides the lymphoid organ spheroids and the gills, the abdominal muscle, eyestalk optic lobe, brains, and the ventral nerve cord were also examined. All the tissues examined, especially in the neural tissues, contained the virus-like particles. The LM and TEM features of the experimentally induced MSGS *P. monodon* were also similar to those of the naturally infected shrimp, suggesting that the 25 nm agent may be a causative virus for MSGS. The virus was also observed under TEM in *P. indicus* and *Macrobrachium rosenbergii* that were co-cultured with MSGS *P. monodon*, although growth retardation of these two species has not been reported. This study showed another example of horizontal transmission of virus from one to the next shrimp species, in which the original

species that were infected by the virus had no clinical manifestation but the species that received the virus showed morbidity.

Key words: Monodon slow growth syndrome - MSGS - growth retardation - *Penaeus monodon* - *P. vannamei* - *P. indicus* - *Macrobrachium rosenbergii* - virus-like particles - cross-species transmission

INTRODUCTION

A relatively sudden, nation-wide occurrence of unusually slow growth and wide variation in size of cultivated black tiger shrimp *Penaeus monodon* has initially reported by shrimp farmers from various growing areas in Thailand since mid 2001. This phenomenon was termed monodon slow growth syndrome or MSGS. After a series of diagnosis of affected shrimp by PCR, RT-PCR, histological methods and electron microscopy, no clear evidence has been found to link MSGS with known pathogenic viruses. It was considered being caused by cryptic pathogen(s) called monodon slow growth agent (MSGA) (1,2). Because the gross appearances are unique and the causative agent is still speculative, case definitions of MSGS shrimp was established for epidermiological purposes (3). The definition of MSGS includes an average daily growth (ADG) at 4 months in culture of less than 0.15 g/day at stocking density of 25 pieces/m^2 or higher, and the coefficient of variation (CV) of the body weight (BW) higher than 35%. Most of the MSGS shrimps have distinctive dark color, with bright yellow/black stripes on the body and appendages. The shrimp used must not be infected by monodon baculovirus (MBV) or hepatopancreatic parvovirus (HPV), which may cause growth retardation (4). Case of growth retardation also had been found in one commercial *P. monodon* farm in East Africa in 2004 (2). After that, a new RNA virus called Laem-Singh virus (LSNV) was described and suspected that could be associated with MSGS. However, it was found in the lymphoid organ and gill specimens of both MSGS ponds and non-MSGS ponds, thus it was not regarded as a possible pathogen (3,5).

During 2002, the MSGS problem, among other problems in shrimp industry, prompted Thai shrimp farmers to switch from farming *P. monodon* to the less problematic white Pacific shrimp *P. vannamei* (6,7). During shifting of the species, co-cultivation of *P. monodon* and *P. vannamei* was widely observed, as well as co-cultivation within the same pond with other less commercially important species, like the fresh water prawn, *Macrobrachium rosenbergii*, the marine shrimp *P. merguensis* and *P. indicus*. The purpose of the co-culturing was to achieve the most production from the same amount of investment provided in mono-culture. With this co-culturing activities, spreading of pathogens across species are expected. As a result, the presence of multiple viruses, parasites and bacterial infections in Thai shrimp industry has been overwhelming as shown by routine histology, polymerase chain reaction (PCR) technique, dot-blot hybridization and *in situ* hybridization assays (1,3,4,8).

*Corresponding author: Tel: (662) 997-2222-30 ext. 1492 Fax (662) 997-2222-30 ext. 1417
E-mail address: ananta_rsu@yahoo.com

Some of these viruses are not known or has not been described in literature. Apparently, except for *P. monodon*, no growth retardation has been reported in other co-cultured species.

The first purpose of this study was to find out if MSGS could be transmitted from the MSGS *P. monodon* and normal *P. vannamei* to healthy *P. monodon*. The second purpose was to find out if the virus found in the lymphoid organ and gill of the MSGS *P. monodon* could also be found in *P. vannamei*, *P. indicus* and *M. rosenbergii* being co-cultured with MSGS *P. monodon*, under conventional transmission electron microscopy (TEM).

MATERIALS AND METHODS

The animal samples studied were 100 MSGS *P. monodon* from a commercial shrimp pond in Thailand. The shrimps clearly demonstrated MSGS by having ADG at 4 months in culture of less than 0.12 g/day, at stocking density of 30 pieces/m^2, and CV of BW at 45%. They had also distinctive body color as described. These MSGS *P. monodon* were co-cultured with *P. vannamei*, which were stocked at 30 pieces/m^2 as well. In addition, samples of 100 *P. vannamei* that were co-cultured with the MSGS *P. monodon*, 100 *P. monodon* from a separate pond that grew normally (ADG at 4 months of >0.2 g/day) and had CV of BW <20%, and 100 *P. vannamei* that were mono-cultured in a separate pond and had no growth retardation, were taken.

Lymphoid organs (LO) from the four groups of shrimps were isolated and all the LO from each group were pooled and homogenized with cold lobster hemolymph buffer (LHB) at a ratio of 1:2 by volume. LHB was prepared using the method described by Paterson & Stewart, 1974 (9). The homogenate was centrifuged and filtered through 0.45 μm membranes and the filtrate was kept at -80°C until use. Either LHB or lymphoid organ extracts (LOE) were injected into the abdominal muscle of *P. monodon*, aged one month, which were divided into 5 groups, 60 animals each. Four groups of these animals were injected with LOE from the four "donors" as described, and one group was injected with LHB. Another 45 one-month old *P. monodon* served as non-injected control group. Each of the first five groups is consisted of 4 replicates, with 15 animals per replicate, and the non-injected control group had 3 replicates. The initial BW of the one-month old *P. monodon* was 0.8-1.0 g and the volume of LOE or LHB injected was 0.1 ml per shrimp. The shrimps in each replicate were reared for 135 days in 1.0m x 1.5m fiberglass tanks containing 0.5m-deep 15ppt seawater. The water was adequately aerated by air stones and the shrimps were given pellet feed twice a day at a rate of 3% BW per day. Water was exchanged at the rate of 30% daily. At the end of the experiment, the shrimps were individually determined for BW, CV of BW and survival rate.

The lymphoid organ, gills, abdominal muscle, eyestalk optic lobe, brains, and the ventral nerve cord of 5 MSGS *P. monodon* (donor) and of 5 *P. monodon* being injected by the LOE (recipient) were examined under TEM. The tissues were fixed in cold 4% glutaraldehyde dissolved in 0.1M Millonig's buffer at pH 7.4 for 6 hours. They were then rinsed in buffer, postfixed in 1% osmium tetroxide for 1 h, washed in Millonig's buffer and dehydrated through a graded series of ethanol, and infiltrated and embedded in Epon-812 or LR-white resins (EMS, Hatfield, PA). The embedded tissues were polymerized in an oven at 70°C for 3 days. Semithin sections were cut, stained with toluidine blue and examined under light microscope (LM). Ultrathin sections were cut and placed on copper grids, contrasted with uranyl acetate and lead citrate for 30 min each and finally examined under a Hitachi (H-7100) TEM.

In a separate experiment, the gill and LO samples were isolated from juvenile *P. vannamei*, *P. indicus* and *M. rosenbergii* that were co-cultured with MSGS *P. monodon* for study under TEM. These specimens were sent from various shrimp farms in central Thailand and East African country.

RESULTS

Light microscopic examination on semithin sections of the MSGS *P. monodon* reveals a number of dark-blue cytoplasmic inclusions as well as pyknotic nuclei and cellular vacuolization in the cells of lymphoid organ spheroid (LOS) **(Figure 1a)**. Whereas, gill, abdominal muscle, brain, eyestalk optic lobe and ventral nerve cord show normal appearance **(Figure 1b-1f)**. Under TEM, several virus-like particles are observed in all the tissues examined. Large electron dense inclusion bodies accompanied by the particles are observed in the cytoplasm of cells in the LOS and the gill filament **(Figure 2a & 2b)**, respectively. The particles are also found at spaces between myofibrils of muscular tissue **(Figure 2c)**. In the brain, the particles are sometimes observed on the cis- and trans-Golgi complex of certain neurons **(Figure 2d)**. In certain cells of the eyestalk optic lobe and ventral nerve cord, the particles are frequently surrounded by double membrane vesicles **(Figure 2e-2g)**, a phenomenon known to exist in certain virus-infected cells (10). Within certain vesicles, the particles are arranged in circular rows as bead-like appearance **(Figure 2e)**, are icosahedral with diameter of 25 nm, and have no envelope **(Figure 2g)**.

Among the four groups of *P. monodon* injected with LOE from different sources, the groups that received LOE from MSGS *P. monodon* and from *P. vannamei* that were co-cultured with MSGS shrimps had a significantly lower BW, compared to the group that received LOE from normal *P. monodon* **(Figure 3)**. High variations in the BW were observed in the two groups, resulting in wide SD and CV; it was noted that not all the injected shrimps had low BW.

When the LO of *P. monodon* that were injected with LOE from MSGS *P. monodon* were examined under LM using semithin sections, it reveals that the LO contain a large number of spheroids, with apoptotic cells and cells with large vacuoles and cytoplasmic inclusions **(Figure 4a)**. The inclusions are of different sizes and colors, some are eosinophilic and the others are basophilic. Some small basophilic inclusions are observed in the LO tubules, but the spheroids distinctively contained higher number of the inclusion bodies. Under TEM, the LO contains the 25 nm icosahedral virus-like particles as well as rod-shaped yellow-head virus (YHV)-like particles **(Figure 4b**-insets), which are 150-800 nm long and 25 nm wide.

The semithin section and ultrastructure of the lymphoid organ of the apparently healthy *P. vannamei* that were co-cultured with MSGS *P. monodon* reveal a large number of spheroids containing 25 nm virus-like particles surrounding cytoplasmic vacuoles, and are associated with inclusion bodies **(Figure 5a & b)**. These features are similar to those found in the lymphoid organ spheroids of MSGS *P. monodon*. The 25 nm virus-like particles are also observed in the pillar cells of the marine shrimp *P. indicus* that had been co-cultured with MSGS *P. monodon* **(Figure 6a)**. Again, the particles are localized loosely around cytoplasmic vacuoles. The virus-like particles are also detected in the cytoplasm of the pillar cell in the gills of *M. rosenbergii* **(Figure 6b)**. However, no cytoplasmic inclusion body as observed in *P. monodon* and *P. vannamei* were observed.

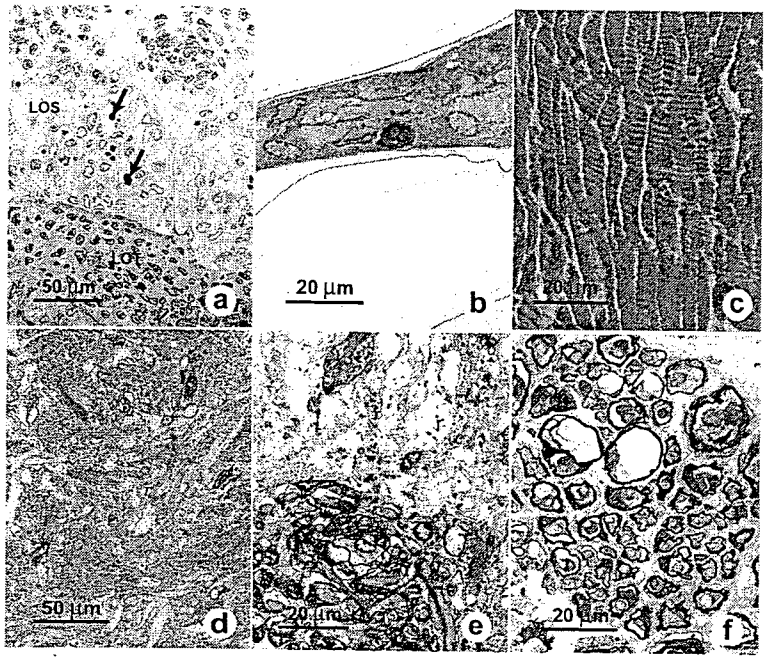

Figure 1. Semithin micrographs of the lymphoid organ (a), gill filament (b), abdominal muscle (c), brain (d), eyestalk (e) and ventral nerve cord (f) of MSGS *P. monodon* with toluidine blue staining. Dark-blue cytoplasmic inclusions (arrows) and cellular vacuolization, features of viral infected cell were observed in the lymphoid organ spheroid (LOS), but not in other tissues. LOT = lymphoid organ tubule.

DISCUSSION

The size of 25 nm virus-like particles found in MSGS *P. monodon* is close to that of infectious hypodermal and hematopoietic necrosis virus (IHHNV), which is 18-22 nm in size. There are, however, lines of evidence that the 25 nm particles in this study are not IHHNV. Firstly, the localization of IHHNV is typically in the nuclear material of the cell (11,12), not in the cytoplasm. Secondly, using polymerase chain reaction (PCR) specific for IHHNV, many MSGS *P. monodon* was found to be negative (1). Another similar virus described previously is the lymphoid organ vacuolizing virus (LOVV) infecting *P. vannamei*; the virus was localized mainly in the cytoplasm and associated with vacuoles in the infected cell (13,14). In certain TEM features, LOVV formed rows of viral particles or bead-like structures, which is similar to the arrangement of the particles described in this study. However, the LOVV is the enveloped virus, its nucleocapsid size is 30 nm and the whole mature virion with envelope is 52-54 nm. Therefore, the particles found in this study are less likely LOVV.

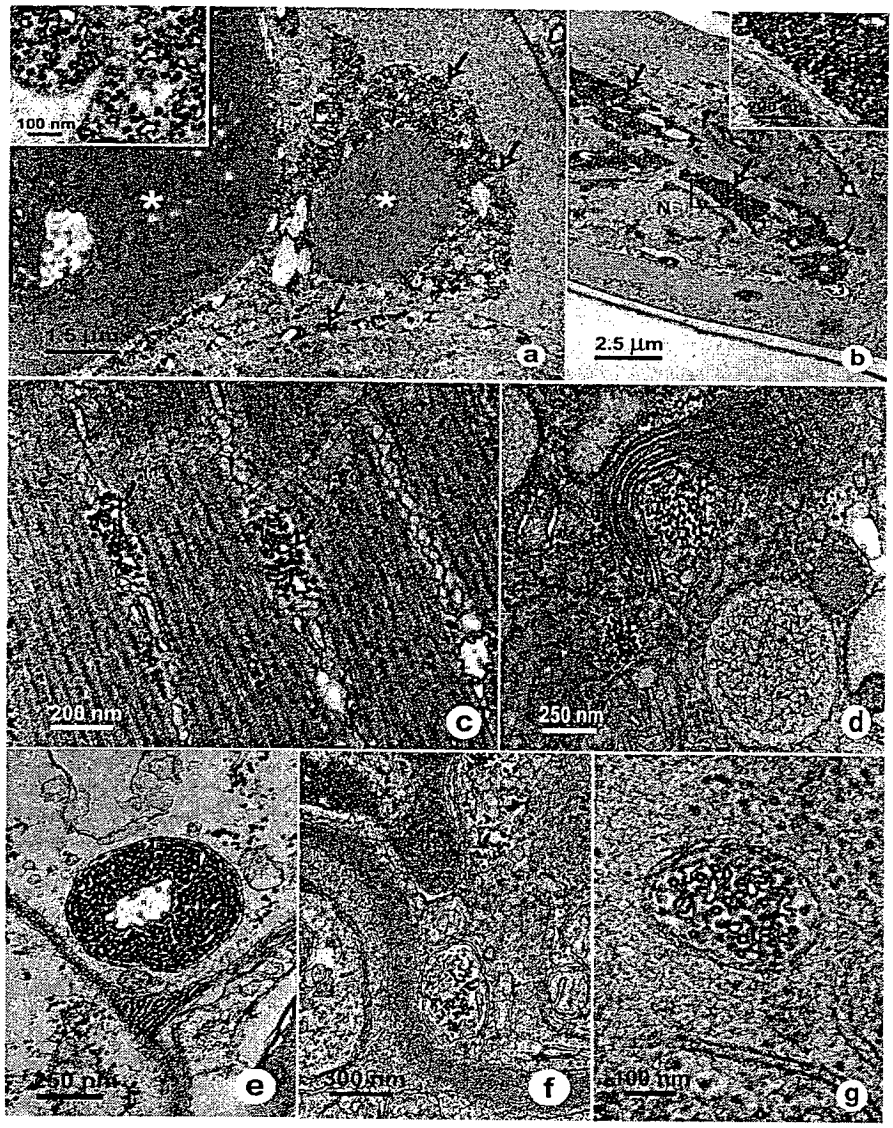

Figure 2. TEM micrographs of MSGS *P. monodon* showing numerous virus-like particles (arrows) in cytoplasm of cell in the lymphoid organ spheroid (a) associated with large electron dense inclusions (*), gill filament (b), muscular tissue (c), brain (d), eyestalk (e) and ventral nerve cord (f and g). The particle size is 25 nm as shown in high magnification in insets and in figure g. In brain (d), virus-like particles (arrows) are found at Golgi complex area of a neuron. The particles in the eyestalk and ventral nerve cord are localized inside membrane-bound vesicles in the cytoplasm (e-g). In the eyestalk, the icosahedral particles are arranged in paracrystaline array or bead-like structure (e). In ventral nerve cord, the particles are observed in cytoplasm of neuroglial cells. At high magnification, the non-enveloped particles in the ventral nerve cord had the size of 25 nm (g).

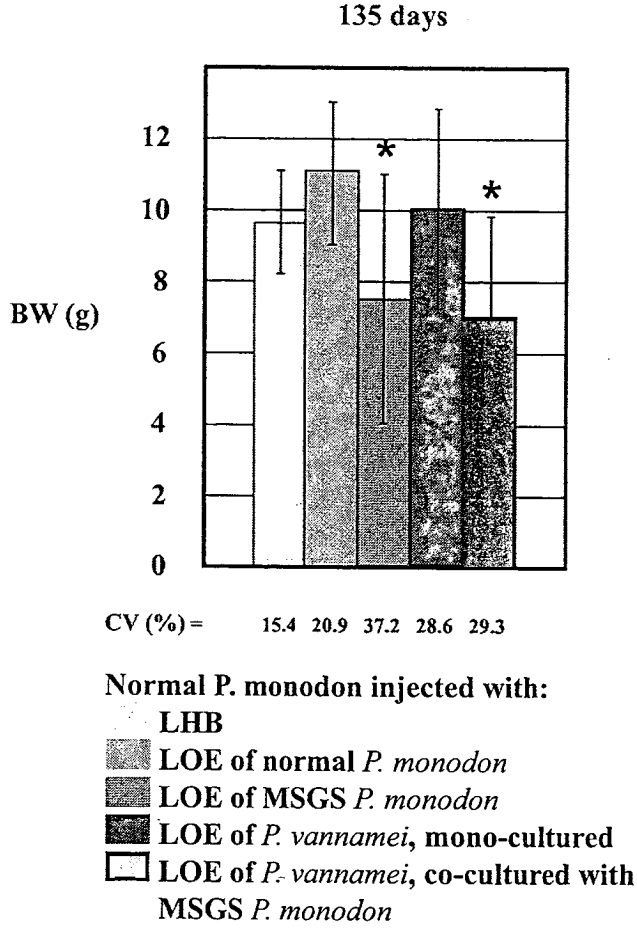

Figure 3. Graph showing mean body weight (BW) and coefficient of variations (CV) of the BW of *P. monodon* at 135 days (4.5 months) after the injections with LHB and lymphoid organ extract (LOE) from two shrimp species from different backgrounds. (*) $P \leq 0.05$, compared to the shrimp injected with LOE from normal *P. monodon*.

Other morphologically similar viruses found in the penaeid shrimps are hepatopancreatic parvovirus (15,16), spawner associated mortality virus, lymphoid organ parvovirus (17,18), Taura syndrome virus (19-22) and infectious myonecrosis virus (23). However, all these viruses exhibit different clinical signs and pathological lesions that are not MSGS feature, they are unlikely to be the same virus as found in this study. The YHV-like particles found in the MSGS *P. monodon* in this study are found to be another type of YHV termed YHV-type4. In field study by RT-PCR, YHV-type4 was found only in 5% of the

MSGS *P. monodon* in Thailand (24). Subsequent results of this non-virulent YHV infection were revealed by RT-PCR test and immunohistochemistry in both normal growth and MSGS ponds; thus it is unlikely that YHV-type4 is the cause of MSGS (25).

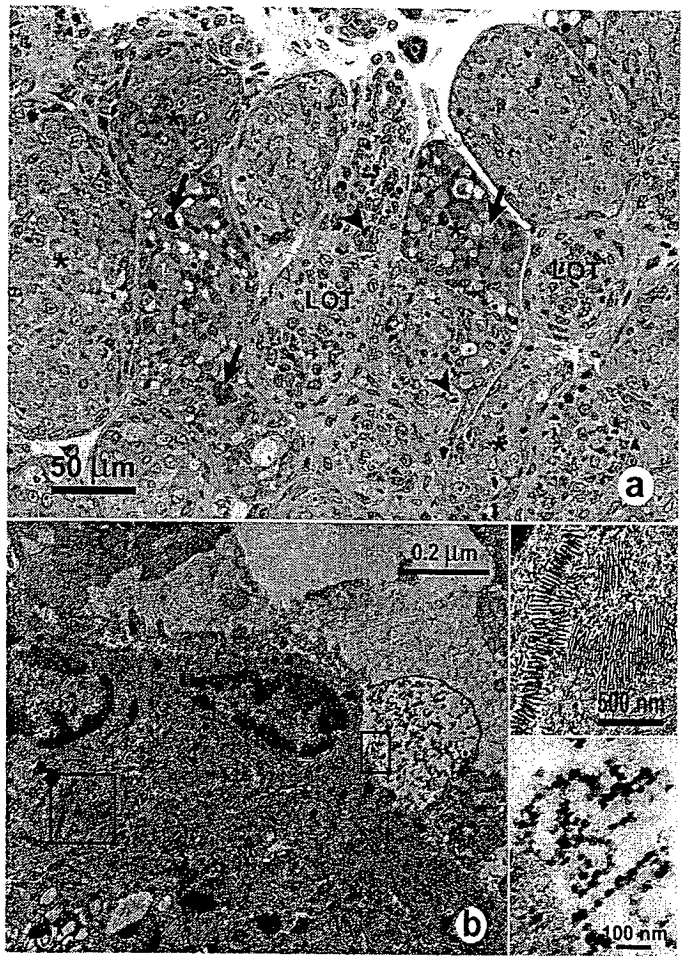

Figure 4. Semithin section stained with toluidine blue (a) and TEM micrograph (b) of the lymphoid organ of *P. monodon* injected by lymphoid organ extract from MSGS *P. monodon*. The semithin section reveals numerous spheroids (*) containing inclusion bodies of different sizes, colors and shapes (arrows); and cytoplasmic vacuoles; as well as fragmented nuclei (arrowheads). LOT = lymphoid organ tubules. Under TEM (b and insets), rod shaped YHV-like particles (120-250 nm in length) and 25 nm icosahedral particles were observed in the cytoplasm of the spheroid cells.

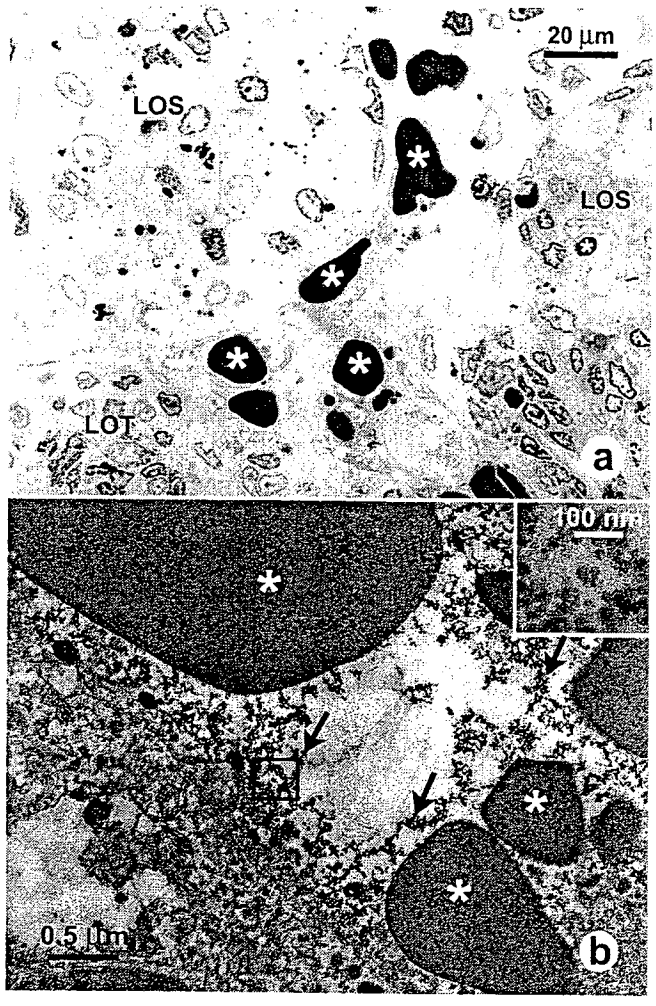

Figure 5. Semithin section with toluidine blue-stain (a) and TEM micrograph (b) of the lymphoid organ of normal *P. vannamei* that were co-cultured with MSGS *P. monodon*. Features of several dark blue inclusions (*) in the cytoplasm of cells of the lymphoid organ spheroids (LOS) were similar to that of in MSGS *P. monodon*. LOT = lymphoid organ tubule. Under TEM, numerous 25 nm virus-like particles (arrows and inset) were observed in vacuolar spaces surrounding electron-dense inclusion bodies (*) within the cytoplasm of the cell.

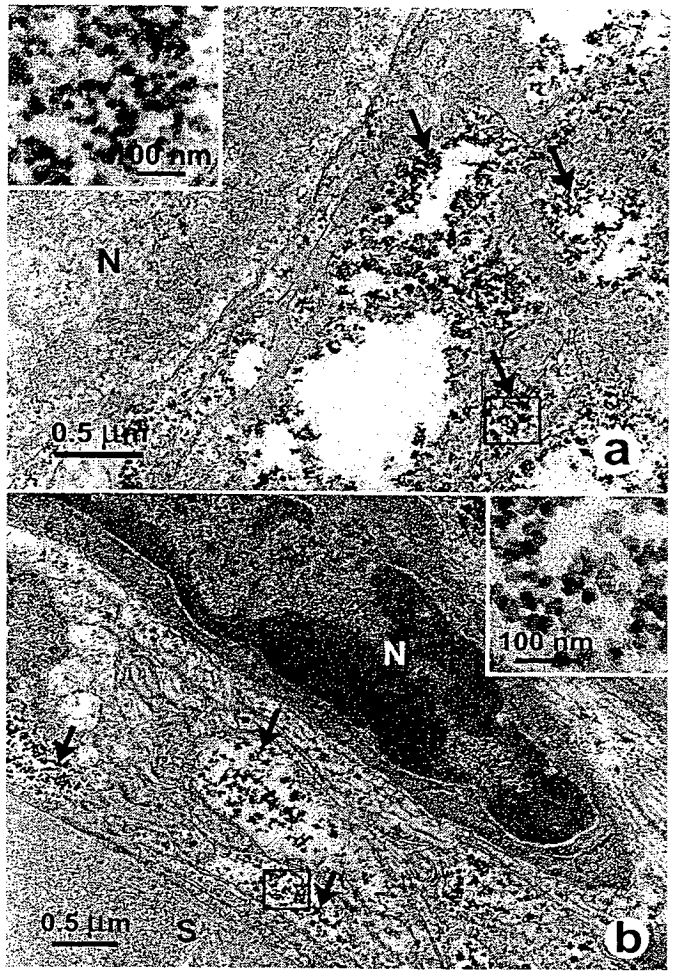

Figure 6. TEM micrographs of the pillar cell of the gill filament of *P. indicus* (a) and the gill filament of *M. rosenbergii* (b) that were co-cultured with MSGS *P. monodon*. Clusters of 25 nm virus-like particles were observed in the cytoplasmic vacuolar spaces (arrows). N = nucleus; S = hemolymph sinus.

In the study by Sritunyalucksana et al. (3), LOE of MSGS *P. monodon* was passed through sucrose gradient ultracentrifugation and a band of unknown virus-like particles of 25 nm size observed under TEM was isolated. The PCR product was absent after the template was treated with RNase; in addition, the RNA-dependent RNA polymerase (RdRp) gene of virus was amplified by reverse transcriptase (RT) PCR; it was thus likely that the particles were RNA virus.

Results of the transmission study revealed that MSGS could be transmitted from MSGS *P. monodon* to healthy *P. monodon* and that LOE from MSGS *P. monodon* contained the causative agent. And it was found that the same type of particles, as observed by TEM, were present in the recipient shrimp. This evidence partially fulfilled Koch's postulate (26,27) since an injection of purified virus particles was not done in this study. An interesting result from this study is that the injection of LOE from *P. vannamei* that were co-cultured with MSGS *P. monodon* also induced MSGS in the recipient shrimp, while *P. vannamei* did not show any clinical manifestation of MSGS. The result suggests that *P. vannamei* might be a carrier of this virus. Not only *P. vannamei*, other kinds of crustaceans such as *P. indicus* and *M. rosenbergii* could also be the carriers of this virus as TEM evidence revealed its presence in these species. It is interesting to test if imported *P. vannamei* broodstocks carry the virus reported in this study.

At this time, the causes that trigger growth retardation are remain to be explored. However, since the nervous system of shrimp, especially the optic lobe has found to control not only in motor and sensory, but also neurohormonal functions, such as molting activity by cells of the X-organ in eyestalk optic lobe, which is directly related to growth and body colors of shrimps and other crustaceans (28). The finding that MSGS *P. monodon* was infected by putative virus in its eyestalk optic lobe and other neural tissues also suggests a potential interference to their neurohormonal regulating system, and possibly resulting in abnormal body color and/or stunt growth. These changing in its body color and slow growth rate are matched to the definition of MSGS shrimps (3). The similar virus-like particles were previously reported in the cytoplasmic area of cells in many tissues derived from embryonic mesoderm of growth-retarded *P. monodon* samples from East Africa (2). Those particles were found associating with unique cytoplasmic lesions in the lymphoid organ and gills. The infected mesodermal and ectodermal tissues included lymphoid organ, gill axis, hemocyte, brain, ventral nerve cord and eyestalk optic lobe. The phenomenon is prevailed in non-virulent honeybee virus infections of nervous tissues, immune cells and neuroendocrine cells that causes paralysis, stunt growth, insufficient metamorphosis of pupae, molting defect and changing of cuticular color of bee colonies (29,30).

In this study, not all the injected shrimps had retarded growth; this may be due to sparing of an infection to the neuroendocrine cells. The finding was similar to clinical symptoms of MSGS in an affected shrimp culturing pond, in which not all the shrimps are growth retarded (3). Another expectation for normal growth shrimps from the same conditions depended on individual shrimps that were capable of their cellular and humoral defenses or may have a specific, or adaptive immune response to viral pathogens as well (31). However, these topics have not been clearly explained by current knowledge.

Finally, the findings suggest a judicious precaution of the new viral transmission among species in alternative rearing and co-cultured practices. This information also suggests that extreme caution should be exercised in the movement of live aquatic stocks destined for aquaculture, especially for co-cultivation. It also points out the importance of constant vigilance of cultivated shrimp and prawn stocks for the possible emergence of new viral pathogens of crustaceans that may be transferred from translocated stocks to native stocks or from native stocks to newly imported exotic species (32,33).

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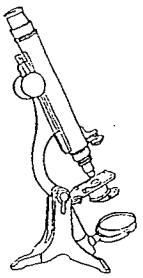

PROCEEDING
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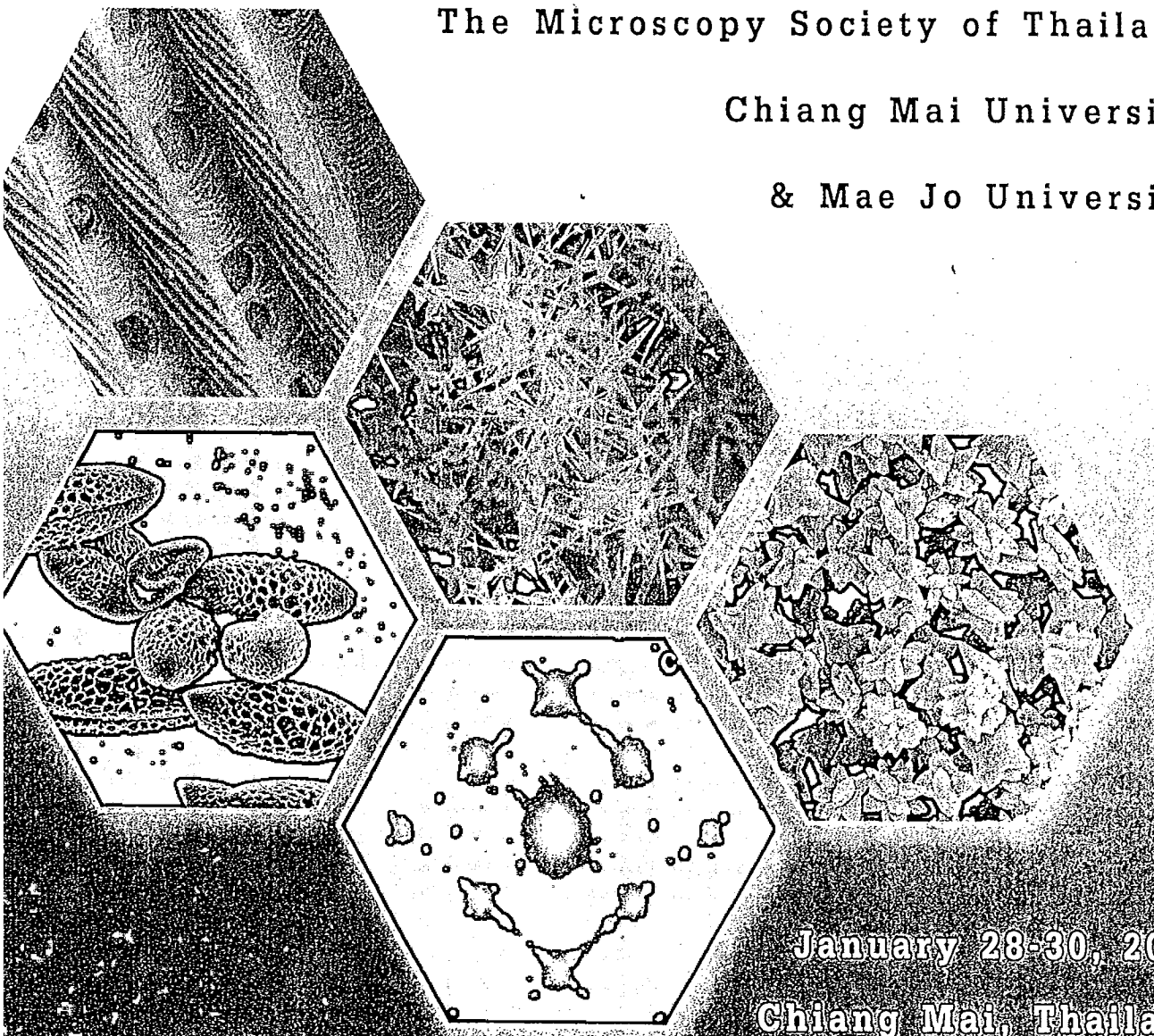

January 28-30, 2009

Chiang Mai, Thailand

Poster Presentation

Re-infection Study of Monodon-slow Growth Virus in *Penaeus monodon* and Other Crustacean Species

Gun Anantasomboon[1,2*], Waraporn Sakaew[2,3], Kanokporn Chayaburakul[1], Boonsirm Withyachumnarnkul[2,3]
[1] Anatomy unit, Department of Medical Science, Faculty of Science, Rangsit University, Patumthani, 12000.
[2] Center of Excellent for Shrimp Biology and Biotechnology (Centex Shrimp) and
[3] Department of Anatomy, Faculty of Science, Mahidol University, Rama 6 Rd. Bangkok 10400, Thailand.
*Corresponding author, e-mail: ananta_rsu@yahoo.com

Abstract

The presence of unique TEM features with numerous virus-like particles and monodon-slow growth syndrome (MSGS) that were inducible in healthy black tiger shrimp, *Penaeus monodon* by re-infection experiments suggest that lymphoid organ extract (LOE), as well as lymphoid organ, gill, muscular, nervous and eyestalk tissues contained monodon-slow growth agent. MSGS may be caused by a new viral agent of approximately 25nm diameter found in tissues derived from embryonic mesoderm and ectoderm. The unique cyto-pathologic lesions associated with the finding virus in other commercial shrimp and prawn species indicate that this new virus could infect many commercial shrimp and prawn species; but not all infected species show clinical manifestations.

Background

In Thailand, a relatively sudden, nation-wide occurrence of unusually slow-growth in the black tiger shrimp, *P. monodon* called Monodon-slow growth syndrome (MSGS) has occurred since 2001. The present study was aimed to find out a putative virus causing MSGS in *P. monodon*. It was major factor causing the majority of Thai shrimp farmers to convert from culturing *P. monodon* to cultivate the Pacific-white shrimp *P. vannamei*. In addition, co-cultivation of *P. monodon*, *P. vannamei*, *P. indicus* and the giant freshwater prawn *Macrobrachium rosenbergii* has been a common practice to make the most production from the same amount of investment providing in mono-culture. With this co-cultivation, transmission of virus(es) among the co-cultured species might be found and is likely facilitate MSGS in shrimp.

Materials and Methods

The first purpose of this study was to find out if MSGS could be transmitted from the MSGS *P. monodon* to healthy *P. monodon*. This was performed by injecting lymphoid organ extracts (LOE) from MSGS shrimp to healthy *P. monodon* and *M. rosenbergii*. To exclude bacteria from the LOE, the extract was filtered through 0.45um membrane before the injection. Mean body weight (BW) and coefficient variation (CV) of BW were determined in the LOE-injected shrimp at 135 days post-injection. The lymphoid organs of the injected shrimp were examined under transmission electron microscopy (TEM). It was also determined for the presence of known shrimp viruses by polymerase chain reaction (PCR) and histological assays using viral detection and typing systems. The second purpose of this study was to find out if the virus found in the lymphoid organ of the injected *P. monodon* is also found in *P. vannamei*, *P. indicus* and *M. rosenbergii* being co-cultured with MSGS *P. monodon* using conventional TEM.

Results, Discussion and Conclusion

Clinical manifestation of MSGS was observed in LOE-injected *P. monodon*. The BW of the injected shrimp was 25-30% lower than control group and showed wide variation in size. Under TEM, lymphoid organs, gills, muscle, brain, eyestalk and ventral nerve cord of the slow-growth shrimp and LOE-injected *P. monodon* contained icosahedral virus particles, 25nm size. No growth retardation was observed in the LOE-injected *M. rosenbergii*, however, the same virus was observed in the gills of the injected prawn. This virus was also observed in the lymphoid organ and gills of *P. vannamei* and *P. indicus* that were co-cultured with MSGS *P. monodon*. The virus particles are usually associated with large electron dense inclusion bodies in the cytoplasm of infected cells, as observed from the semi-thin sections.

Results suggest that MSGS is probably caused by an infection with the 25nm virus. This virus is transmissible among all four crustacean species under study, however, only *P. monodon* show clinical manifestation of MSGS. With the unique pathological lesion and the negative results from PCR assays to determine the presence of any known shrimp viruses in the MSGS samples, it is most likely this 25nm virus is a new shrimp virus, a putative virus causing MSGS.

Acknowledgements

This study was funded by grant no. MRG5180212 from the Thailand Research Fund (TRF) and the Centex Shrimp, Mahidol University.

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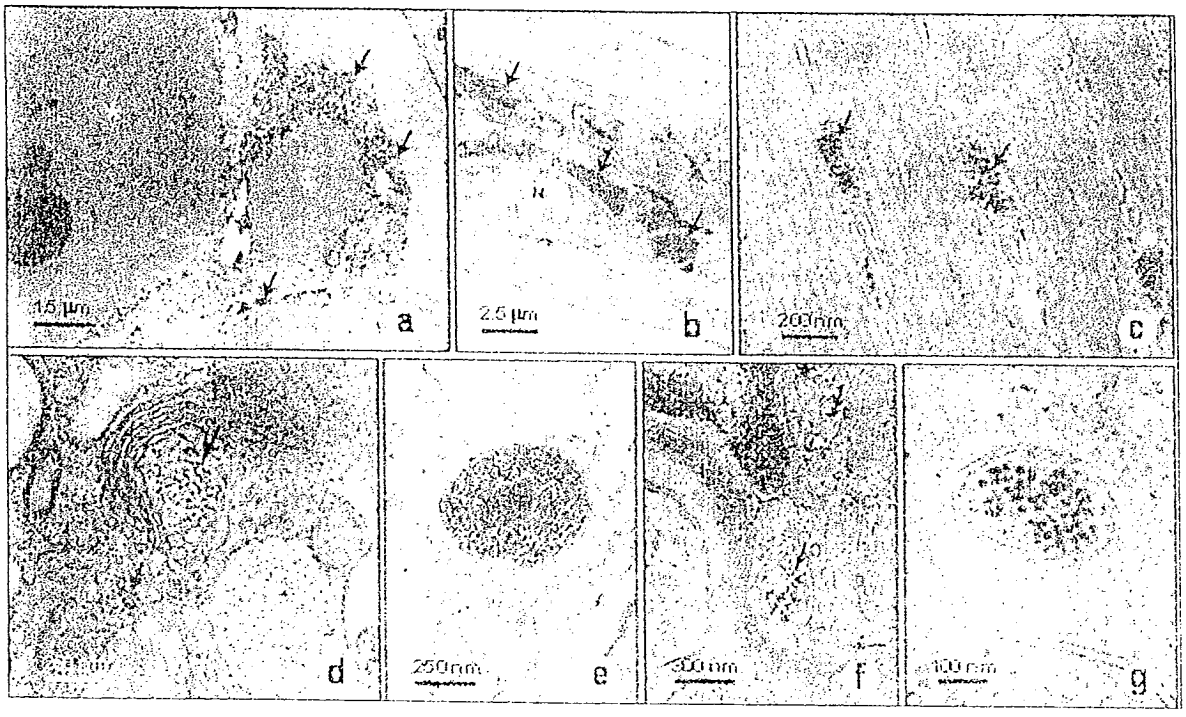

Fig.1 TEM features of MSGS *P. monodon* showing numerous virus-like particles (arrows) in cytoplasm of cells in the lymphoid organ spheroid (a) associated with large electron dense inclusions (*), gill axis (b), muscular tissue (c), brain (d), eyestalk (e) and ventral nerve cord (f). Under high magnification, the particles with 25nm diameter are icosahedral and have no envelope (g).

RE-INFECTION STUDY OF MONODON-SLOW GROWTH VIRUS IN *Penaeus monodon* AND OTHER CRUSTACEAN SPECIES

Gun Anantasomboon[1,2*], Kanokporn Chayaburakul[1,2], Waraporn Sakeaw[3] and Boonsirm Withyachumnarnkul[2,3]

[1]*Anatomy Unit, Department of Medical Science, Rangsit University, Patum-thani 12000,*
[2]*Center of Excellent for Shrimp Biology and Biotechnology (Centex Shrimp), and*
[3]*Department of Anatomy, Faculty of Science, Mahidol University, Bangkok 10400, Thailand.*
**E-mail: ananta_rsu@yahoo.com*

tract The presence of unique TEM features with numerous virus-like particles and monodon-slow growth syndrome (MSGS) that were inducible in hy *P. monodon* by re-infection experiments suggest that lymphoid organ extracts (LOE), as well as lymphoid organ, gill, muscular tissue, nervous e and eyestalk contained monodon-slow growth agent. MSGS may be caused by a new viral agent of approximately 25 nm diameter found in tissues ed from embryonic mesoderm and ectoderm. The unique cyto-pathologic lesions associated with the finding virus in commercial shrimp and prawn es indicate that this new virus could infect many commercial shrimp and prawn species; but not all infected species show clinical manifestations.

ords: *Penaeus monodon*, TEM, virus, inclusion body Monodon-slow growth syndrome (MSGS)

duction

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tives

o determine the cause of MSGS.
o determine tissue tropism of MSGA.
o elucidate transmission of MSGA among ommercial shrimp and prawn species.

ials and Methods

Transmission

d organs were isolated from MSG *P. monodon*, *monodon*, normal *L. vannamei* that were co-culture G *P. monodon* and *L. vannamei* that were reared y. The four groups of lymphoid organs were y homogenized with cold lobster hemolymph buffer [1:2), centrifuged and filtered through 0.45 µm es and kept in –80°C before use. These lymphoid tracts (LOE) were separately injected into 60 healthy *lon* as 15 shrimp/group, aged of 1 month and 0.8-1 g ight (BW), at 0.1 ml/shrimp. The injected shrimp red separately for 135 days in 1-ton concrete tanks ation and with approximately 12:12 h light/dark iods. Commercial pellets were given 2-3 times a day 0-30% of water was exchanged daily. At the end of riment, the shrimp were determined for mean body W) and coefficient variation (CV) of BW. The LOE injected into 15 pieces of *M. rosenbergii* with 10 g the prawn were observed for another 12 days.

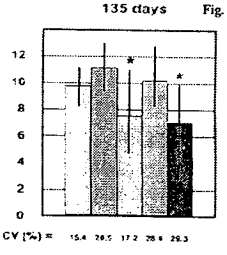

Fig. 3 Graph showing mean body weights of *P. monodon* at 135 days after injected with:
- Lobster hemolymph buffer (LHB)
- LOEs from normal *P. monodon*
- MSG *P. monodon*
- *L. vannamei* that were reared in a separate pond
- *L. vannamei* that were co-cultured with MSG *P. monodon*

CV = coefficient of variation of body weight (BW).
*$P \leq 0.05$, compared to the shrimp injected with LHB.

s Revealed Under Transmission Electron Microscope

rate experiment, several tissue samples were isolated from certain shrimp and prawn or study under transmission electron microscopy (TEM). The tissues included organs, gills, abdominal muscle, eyestalks, brains, and ventral nerve cords from MSG *on*; lymphoid organs and gills of *L. vannamei* and *P. indicus* that were co-cultured *P. monodon*; and gills of LOE-injected *M. rosenbergii*. The tissues were fixed in glutaraldehyde dissolved in 0.1M Millonig's buffer at pH 7.4 for 6 h. The tissues were d in buffer, post-fixed in 1% osmium tetroxide for 1 h, washed in Millonig's buffer drated through a graded series of ethanol, and subsqeuently embedded in Epon-812 IS, USA). The embedded tissues were polymerized in an oven at 70°C for 3 days. sections were cut, stained with toluidine blue and examined under light microscope ltrathin sections were contrasted with uranyl acetate and lead citrate for 30 min each ined by a Hitashi (H-7100) TEM.

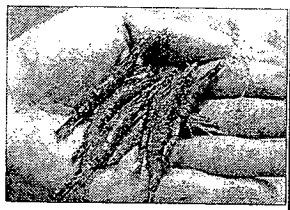

Fig. 1 Photograph of slow-growth *P. monodon* with body weight of only 3-5 g at two months in culturing pond.

Fig. 2 Provinces in Thailand where the new virus in cultivated *P. monodon* กุ้งกุลา wild broodstocks were noticed during the year 2003-2005.

Discussion

The unique TEM features of virus-like particles and the finding that MSGS was inducible in healthy *P. monodon* by injection with LOE suggest that MSGA could be a new virus with 25 nm in size. However, classification of the new viral agent would require characterization in its nucleic acid. This virus could infect many commercial shrimp and prawn species, but not all infected species show clinical manifestations. Surprisingly, even in *P. monodon*, not all the shrimp infected with this virus, even from the same pond or from the same environment, developed clinical symptom of slow growth. This phenomenon suggests that the symptom of MSGS is induced by certain interaction between the shrimp and the virus. Evidence from the field also showed that shrimp of different families showed different manifestation in the MSG pond, there were a family that had mass mortality, a family with MSGS and a family with normal growth (Withyachumnarnkul, unpublished). Therefore, shrimp genetics may play role in the response to this new virus, and because of the wide distribution of the virus, wild population of *P. monodon* may have been naturally selected and certain families may have to trade it with slow growth. Moreover, this findings also suggest a precaution of the new viral transmission among species in alternative rearing and co-cultured practices.

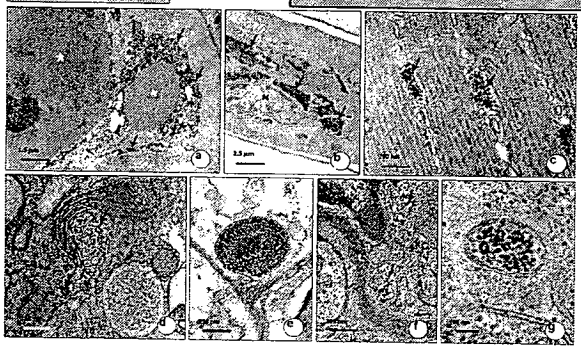

Fig. 4 TEM features of MSG *P. monodon* showing numerous virus-like particles (arrows) in cytoplasm of cell in the lymphoid organ spheroid (a) associated with large electron dense inclusions (*), gill axis (b), muscular tissue (c), brain (d), eyestalk (e) and ventral nerve cord (f). Under high magnification, the particles, with 25 nm diameter, were icosahedral shape and had no envelope (g).

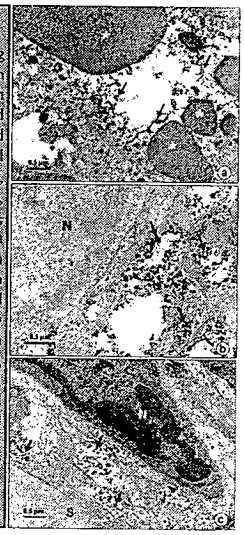

Fig. 5 TEM micrograph of cells in lymphoid organ spheroid of *L. vannamei* (a); gill axis cell of *P. indicus* (b) that were co-cultured with MSG *P. monodon*; and gill tissue of LOE-injected *M. rosenbergii* (c) from MSG *P. monodon* featuring numerous small virus-like particles at 25 nm diameter (arrows). These particles are usually deposited in the cytoplasmic vacuolar spaces surrounding the electron-dense inclusion bodies (*). N = nucleus that appeared normal with chromatin condensation; S = hemolymph sinus.

Results

Lymphoid organ extracts (LOE) from MSG *P. monodon* and from *L. vannamei* that were co-cultured with MSG *P. monodon* caused slow growth in healthy *P. monodon*. Mean BW of the LOE-injected shrimp was 20-30% lower than LHB-injected group, with LOE from normal *P. monodon*, or with LOE from *L. vannamei* that were separately reared (Fig. 3). Under TEM, the lymphoid organ, gill, muscular tissue, brain, eyestalk and ventral nerve cord of MSG *P. monodon* (Fig. 4a-f) as well as normal *P. monodon* in the same pond contained icosahedral virus-like particles without envelope (~25 nm diameter) (Fig. 4g, at high magnification). In addition, the same features were observed in many cells of lymphoid organ spheroid of *L. vannamei* (Fig. 5a) and in the gill axis cells of *P. indicus* (Fig. 5b) that were co-cultured with MSG *P. monodon*. The virus-like particles were also detected in gills of the LOE-injected *M. rosenbergii* (Fig. 5c). From ultrathin sections (Fig. 6a), it was observed that these viral particles are usually associated with large electron-dense inclusion bodies (*) as observed in semithin sections with toluidine blue stain (Fig. 6b).

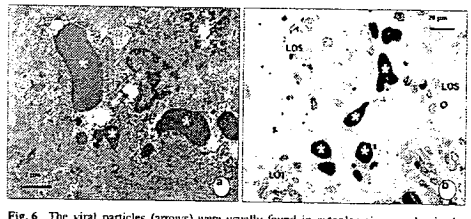

Fig. 6 The viral particles (arrows) were usually found in cytoplasmic vacuoles in the lymphoid organ (a) and were associated with large electron dense inclusion bodies (*) in the infected shrimp. This unique lesions correspond to dark blue inclusions (*) observed in semi-thin sections with toluidine blue-stain (b). LOS = lymphoid organ spheroid; LOT = lymphoid organ tubule.

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Acknowledgements

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ดย่อ

สนอผลงานแบบโปสเตอร์

ชุมนักวิจัยรุ่นใหม่ พบ เมธีวิจัยอาวุโส สกว.

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PJ-BIO-F25

Injection of dsRNA Specific to Non-coding Sequences of Yellow-head Virus (YHV) Genome Induces Protection of Penaeid Shrimp from Mortality and Reduces YHV Multiplication

Anantasomboon, G.[1,2*], Saenapin, S.[2], Browdy, C. L.[3], Withyachumnarnkul, B.[2,4], Flegel, T. W.[2,5]

[1]*Anatomy Unit, Department of Medical Science, Faculty of Science, Rangsit University, Pathum-thani 12000, Thailand*
[2]*Centex Shrimp, Faculty of Science, Mahidol University, Rajthevi, Bangkok 10400, Thailand*
[3]*Marine Infectious Laboratory, South Carolina Department of Natural Resources, SC, USA*
[4]*Department of Anatomy and* [5]*Department of Biotechnology, Faculty of Science, Mahidol University, Bangkok 10400, Thailand*

Abstract

Yellow-head virus (YHV) is an invertebrate RNA virus in order *Nidovirales* that causes mass mortality called yellow-head disease (YHD) in penaeid shrimp. The original YHV outbreaks occurred in Thailand in the early 1990's. At present, 5 genetic variants are known in the YHV complex, but only the original YHV-type 1 variant appears to be highly virulent. By injecting dsRNA preparations based on non-coding sequence of ORF1b and ORF3 regions of the YHV genome into 1g Pacific whiteleg shrimp *Penaeus (Litopenaeus) vannamei* challenged with a 90% lethal dose of YHV inoculum we were able to demonstrate sequence-specific antiviral protection when compared untreated control shrimp (Fig. 1). At 14 days post-challenge the dsRNA treated shrimp survival was 67% with the ORF3 preparation and 48% with the ORF1b preparation compared to 90% survival for the control shrimp. Although survivors were grossly normal, all were positive for YHV-type 1 by reverse transcriptase polymerase chain reaction (RT-PCR) assay specific for the ORF1b sequence of YHV. Real-time PCR quantification of YHV copies prepared from gill tissues of RNA interference (RNAi) shrimps showed lower copy number compared to moribund shrimp. Positive immunohistochemical reactions for YHV structural protein gp116 in the lymphoid organ and gill tissues confirmed the presence of YHV. On the other hand, immunofluorescent signal lower in the surviving shrimp than in the moribund control shrimp, indicating lower viral loads in the former. These results suggested that the surviving shrimp were tolerant rather than resistant to YHV-1 infection since RNAi was not associated with absence of YHV.

Keywords: dsRNA, RNAi, virulent virus, yellow-head virus, YHV-1, penaeid shrimp, yellow-head disease (YHD), lethal dose, viral tolerant, SPR shrimp, SPF shrimp

Outputs

1. Gangnonngiw W, Anantasomboon G, Sang-oum W, Sriurairatana S, Sritunyalucksana K, Flegel TW. *Non-virulence of a recombinant shrimp nidovirus is associated with its non structural gene sequence and not a large structural gene deletion.* Virology **2009**; 385: 161-168.

*Corresponding author.
Tel.: 0-2997-2222 ext. 1492; Fax: 0-2997-2222 ext. 1417
E-mail: ananta_rsu@yahoo.com

INJECTION OF dsRNA SPECIFIC TO NON-CODING SEQUENCES OF YELLOW-HEAD VIRUS (YHV) GENOME INDUCES PROTECTION OF PENAEID SHRIMP FROM MORTALITY AND REDUCES YHV MULTIPLICATION

Gun Anantasomboon[1,2*], Saengchan Senapin[2,3], Craig L Browdy[4], Boonsirm Withyachumnarnkul[2] and Timothy W Flegel[2]

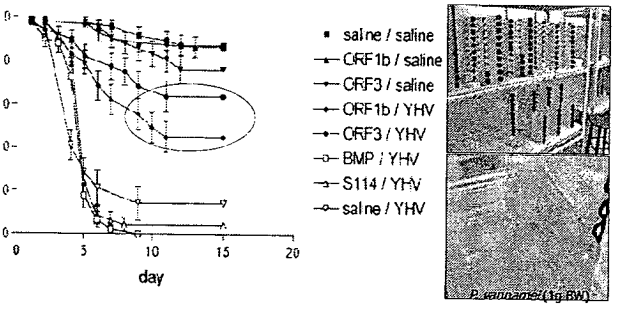
[1]Anatomy Unit, Department of Medical Science, Faculty of Science, Rangsit University, Patumthani 12000;
[2]Centex Shrimp and Department of Anatomy, Faculty of Science, Mahidol University, Bangkok 10400;
[3]BIOTEC, Ministry of Science and Technology, Patumthani 12120; Thailand
[4]South Carolina Department of Natural Resources (SCDNR) and Hollings Marine Laboratory, SC, 29412 USA.
**e-mail address:* ananta_gun@yahoo.com

roduction

ow-head virus (YHV) is an invertebrate RNA virus in order *Nidovirales* causes mass mortality in penaeid shrimp called yellow-head disease D). The original YHV outbreaks occurred in Thailand and Taiwan in early 1990's. At present, 5 genetic variants are known in the YHV plex, but only the original YHV-type 1 variant appears to be highly ent. Most studies have focused on acute type 1 viral infections, ever, there is limited data on survivors of these outbreaks and RNA ference (RNAi) eliminating the YHV.

erials, Methods and Results

we compared 1g surviving and moribund Pacific white-leg shrimp *Penaeus enaeus) vannamei* that were injected with dsRNA into 3rd-4th abdominal muscle, ining with 90% lethal dose (LD90) of YHV inoculum at day 2 later. By injecting As preparations based on ORF1b and ORF3 regions of the YHV genome, result nstrate that non-coding dsRNAs designed from ORF1b and ORF3 regions induce ence-specific antiviral protection when compared with untreated control shrimp 1). At 15 days post-challenge the dsRNA treated shrimp survival was 67% with RF3 (ORF3/YHV) preparation and 48% with the ORF1b (ORF1b/YHV) preparation ared to low survival rate of 14% for the untreated control shrimp. Although rors were grossly normal, but all were positive for YHV-type1 by reverse criptase polymerase chain reaction (RT-PCR) assay specific for the ORF3 nce of YHV (Fig. 2). The 739 bp amplicon from gill samples of surviving and und shrimp was amplified and demonstrated in agarose gel photograph. ve immunohistochemical reactions for YHV structural protein, gp116 using V3-2B clonal antibody (Sithigorngul, 2000; 2002) in the lymphoid organ (LO) confirmed resence of YHV. However, the immunofluorescent signal was lower in the RNAi-ing shrimps correlated to lower copy number of YHV genome than in moribund ol shrimp by real-time PCR quantification (data not show).



1. Graph showing percent survival of shrimp treated or not with dsRNAs. At day 15 post-administration with dsRNAs and an LD90 dose of YHV later, SPF *Penaeus vannamei* injected with dsRNA specific to sequences of YHV-ORF1b and –ORF3 showed significant levels of protection (48% survival with ORF1b/YHV (♦) dsRNA and 67% survival with ORF3/YHV (●) dsRNA) against mortality from yellow-head disease compared to result from other control groups: saline/saline injection (■); ORF1b dsRNA/negative YHV (▲); ORF3 dsRNA/negative YHV (▼); non-shrimp dsRNA(BMP)/YHV (□); catfish dsRNA(S114)/YHV (Δ); and saline/positive YHV (▽).

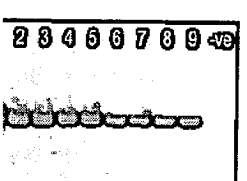


Figure 2. Ethidium bromide stained electrophoresis gel showing positive semi-quantitative RT-PCR results of RNA extracts from gills of control shrimp and shrimp from dsRNAs treated groups. The primers for 739 amplicon was designed from ORF3 region of the YHV and used for RT-PCR analysis at 35 cycles. Lane M, 100 bp DNA standard marker; Lanes 1-5, example of moribund shrimp at day 2 from positive YHV (▽); Lanes 6-9, surviving shrimp from dsRNA treated groups: 6 (♦), 7 (●), 8 (□), 9 (Δ); Lane -ve, negative control shrimp (■) result at d5. Note that all of the bands for survivors are less intense than those of moribund shrimp.

Objectives

The purpose of this study is to examine survivors of a YHV-type 1 challenge test combining with RNA interference (RNAi) to YHV infection in shrimp and to determine whether or not they were infected with YHV-1 and if so to determine some characteristics of the infection in terms of survival rate, histopathology, copy number of YHV replications and immunohistochemical characteristics of the virus present.

Conclusions and Discussion

The results indicate that RNAi using dsRNAs prepared from non-coding sequences from ORF3 and ORF1b regions could protect shrimp from mortality at high dose (LD90) of YHV injection and reduces YHV multiplication. However, surviving shrimp from dsRNA treated groups were positive YHV-type 1 infection by semi-quantitative RT-PCR analysis. Furthermore, surviving shrimp was related to low YHV copy number in the former and low amount of YHV-structural proteins, gp116, when compared to the moribund shrimp by real-time PCR and immunofluorescent detection. These results suggest that the surviving shrimp appear to be tolerant rather than resistant to YHV-1 infection since RNAi was not associated with absence of YHV. It is also hypothesized that RNAi generated by the dsRNAs tested partially blocked the synthesis of viral proteins which may have showed but not eliminated viral replication.

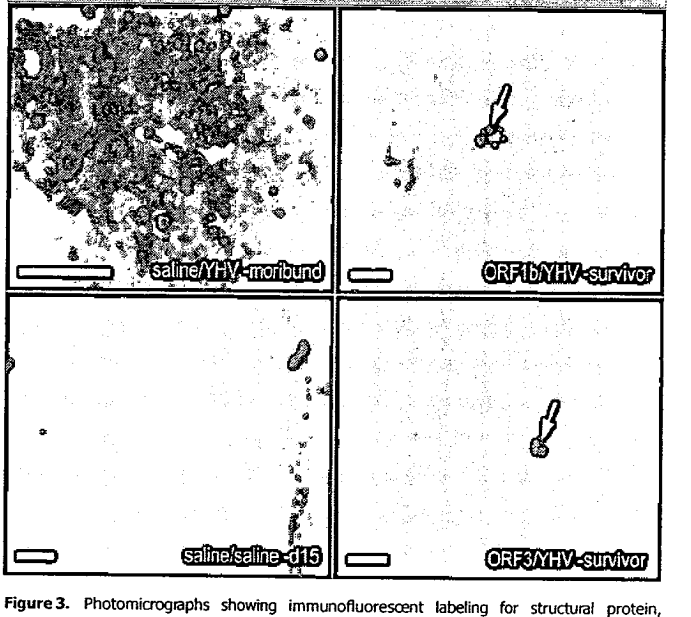


Figure 3. Photomicrographs showing immunofluorescent labeling for structural protein, gp116 of YHV in the lymphoid organ (LO) sections from experimental *P. vannamei*. Immunofluorescent signals (yellow-green color) of moribund shrimp at day 2 after YHV injection (saline/YHV) are intensely observed in the LO tubular (LOT) matrices. Whereas, immuno-reactions in surviving shrimp treated with dsRNAs (ORF1b/YHV & ORF3/YHV) reveal mildly positive signal with some infected cells at clusters of aggregated hemocytes (arrows), but are very faint to negative in the LOT. Bar = 50 μm.

Acknowledgements

This study was supported by research grants from the Thailand Research Fund (TRF: No. MRG5180212) and the Centex Shrimp, Mahidol University, Bangkok, Thailand. The authors would like to thank Dr Enrique de La Vega, Dr Javier Robalino, postdoctoral fellow from Medical University of South Carolina, Caroline Payne, Deil L and Austin Hughes from Hollings Marine Laboratory, Charleston, SC for information regarding GAV, TSV-RNAi research and their help.

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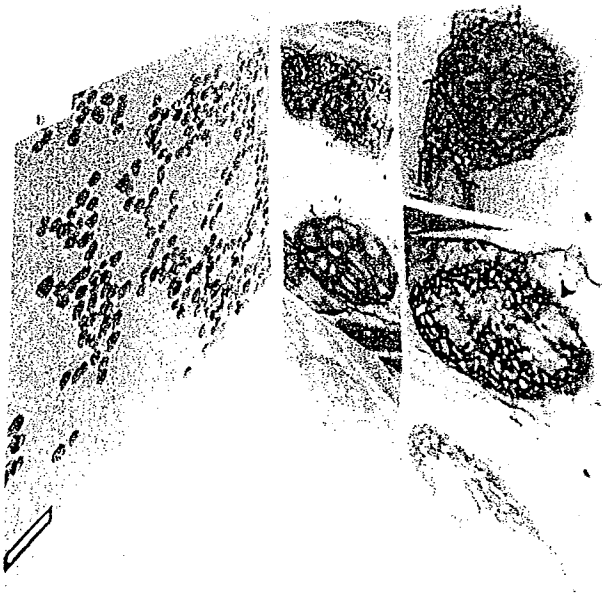

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Oral Presentation

Injection of dsRNA Specific to Non-coding Sequences of Yellow-head Virus (YHV) Genome Induces Protection of Penaeid Shrimp from Mortality and Reduces YHV Multiplication

Gun Anantasomboon[1,2*], Saengchan Saenapin[2], C.L. Browdy[3], Boonsirm Withyachumnarnkul[2,4], Timothy W. Flegel[2]
[1] Anatomy unit, Department of Medical Science, Faculty of Science, Rangsit University, Patumthani, 12000.
[2] Center of Excellent for Shrimp Biology and Biotechnology (Centex Shrimp), Mahidol University, Thailand.
[3] South Carolina Department of Natural Resources and Holling's Marine Laboratory, Charleston, SC, USA.
[4] Department of Anatomy, Faculty of Science, Mahidol University, Rama 6 Rd. Bangkok 10400, Thailand.
*Corresponding author, e-mail: ananta_rsu@yahoo.com

Abstract

Yellow-head virus (YHV) is an invertebrate RNA virus in order *Nidovirales* that causes mass mortality in penaeid shrimp called yellow-head disease (YHD). The original YHV outbreaks occurred in Thailand in the early 1990's. At present, 5 genetic variants are known in the YHV complex, but only the original YHV-type 1 variant appears to be highly virulent. By injecting dsRNA preparations based on non-coding sequence of the ORF1b and ORF3 regions of YHV genome into Pacific whiteleg shrimp *Penaeus (Litopenaeus) vannamei* (1g) challenged with a 90% lethal dose of YHV inoculums, we were able to demonstrate sequence-specific antiviral protection when compared to the untreated control shrimp (Fig. 1). At 14 days post-challenge, the survival of dsRNA treated shrimp was 67% with the ORF3 preparation and 48% with the ORF1b preparation when compared to 90% survival of the control shrimp. Although grossly normal, all survivors were positive for the YHV-type 1 by reverse transcriptase polymerase chain reaction (RT-PCR) assay specific for the ORF1b sequence of YHV. Real-time PCR quantification of YHV copies prepared from the gill tissues of RNA interference (RNAi) shrimps showed lower copy number than moribund shrimp. Positive immunohistochemical reactions for the YHV structural protein gp116 in the lymphoid organs and gill tissues confirmed the presence of YHV. On the other hand, the less immunofluorescent signal detected in the surviving shrimp than in the moribund control shrimp, indicating lower viral loads in the former. These results suggested that the surviving shrimp appeared to be tolerant rather than resistant to YHV-1 infection since RNAi was not associated with the absence of YHV.

Background

Yellow-head virus (YHV) is an invertebrate RNA virus in order *Nidovirales* that causes mass mortality in penaeid shrimp called yellow-head disease (YHD). The original YHV outbreak had been found in Thailand and Taiwan since the early 1990's. At present, 5 genetic variants are known in the YHV complex, but only the original YHV-type 1 variant appears to be highly virulent. Most studies have focused on acute type 1 viral infections, however, there is limited data on survivors of these outbreaks and RNA interference (RNAi) eliminating the YHV.

Objectives, Materials and Methods

The purpose of this study is to examine survivors of a YHV-type 1 challenge test combining with RNA interference (RNAi) to YHV infection in shrimp, to determine whether or not they were infected with YHV-1, and to determine some characteristics of the infection including histopathology, copy number of YHV replications in shrimp tissues by real-time PCR quantification and immunohistochemical localization of the virus using confocal laser scanning microscopy (CLSM) and conventional TEM.

Results

By injecting dsRNAs preparations based on the ORF1b and ORF3 regions of YHV genome, result demonstrate that non-coding dsRNAs designed from the ORF1b and ORF3 regions induce sequence-specific antiviral protection when compared to untreated control shrimp. At 15 days post-challenge, the dsRNA treated shrimp survival was 67% with the ORF3 (ORF3/YHV) preparation and 48% with the ORF1b (ORF1b/YHV) preparation when compared to low survival rate (14%) for the untreated control shrimp. Although survivors were grossly normal, but all were positive for YHV-type 1 by reverse transcriptase polymerase chain reaction (RT-PCR) assay specific for the ORF3 sequence of YHV. The 739 bp amplicon from gill samples of surviving and

moribund shrimp was amplified and demonstrated by photography of the stained agarose gel. Positive immunohistochemical reactions for YHV structural protein, gp116 using V3-2B monoclonal antibody (Sithigorngul, 2000; 2002) in the lymphoid organs (LO) confirmed the presence of YHV. However, the immunofluorescent signal was lower in the RNAi-surviving shrimp correlated to lower copy number of YHV genome than in moribund control shrimp by real-time PCR quantification.

Discussion and Conclusions

The results indicate that RNAi using dsRNAs prepared from non-coding sequences from ORF3 and ORF1b regions could protect shrimp from mortality at high dose (LD90) of YHV injection and reduce YHV multiplication. However, surviving shrimp from dsRNA treated groups were positive YHV-type 1 infection by semi-quantitative RT-PCR analysis. Furthermore, surviving shrimp was related to low YHV copy number in the former and low amount of YHV-structural proteins, gp116, when compared to the moribund shrimp by real-time PCR and immunofluorescent detection. These results suggest that the surviving shrimp appears to be tolerant rather than resistant to YHV-1 infection since RNAi was not associated with the absence of YHV.

It is also hypothesized that RNAi generated by the dsRNAs tested partially blocked the synthesis of viral proteins which may have showed but not eliminated viral replication.

Acknowledgements

This study was funded by the Thailand Research Fund (TRF): grant no. MRG5180212 and the Centex Shrimp, Mahidol University.

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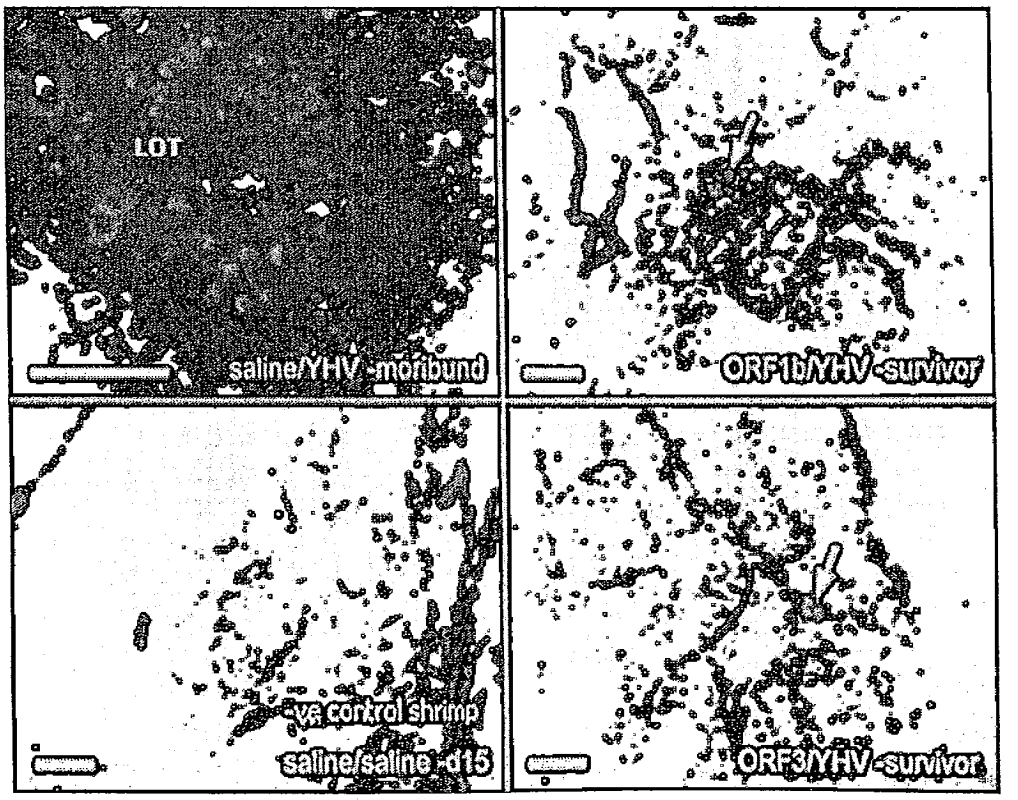

Figure 1 Photomicrographs showing immunofluorescent labeling of structural protein, gp116 of YHV in the lymphoid organ (LO) sections from experimental *P. vannamei*. Immunofluorescent signals (yellow-green color) of moribund shrimp at day 2 after YHV injection (saline/YHV) are intensely observed in the LO tubular (LOT) matrices. Whereas, the immuno-reactions in surviving shrimp treated with dsRNAs (ORF1b/YHV & ORF3/YHV) reveal mildly positive signal with some infected cells at clusters of aggregated hemocytes (arrows), but are very faint to negative in the LOT. Bar = 50 mm.

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สำนักงานกองทุนสนับสนุนการวิจัย
The Thailand Research Fund

สำนักงานคณะกรรมการการอุดมศึกษา
Commission on Higher Education

Protection of Penaeid Shrimp from Mortality and Reduces YHV Multiplication Using RNAi Technology Specific to non-coding Fragments of YHV Genome

Anantasomboon, G.[1,2*], Saenapin, S.[2], Browdy, C. L.[3], Withyachumnarnkul, B.[2,4], Flegel, T. W.[2,5]

[1]*Anatomy Unit, Department of Medical Science, Faculty of Science, Rangsit University, Pathum-thani 12000, Thailand*
[2]*Centex Shrimp, Faculty of Science, Mahidol University, Rajthevi, Bangkok 10400, Thailand*
[3]*Marine Infectious Laboratory, South Carolina Department of Natural Resources, SC, USA*
[4]*Department of Anatomy and* [5]*Department of Biotechnology, Faculty of Science, Mahidol University, Bangkok 10400, Thailand*

Abstract

The original yellow-head virus (YHV-type 1) outbreaks have occurred in Thailand since the early 1990's and cause continue mass mortalities in penaeid shrimp called yellow-head disease (YHD). Lacking of adaptive immunity in shrimp, it had been believed that RNA interference/or inhibition (RNAi) could eliminate RNA viruses from infected animal. We applied RNAi technology to treat penaied shrimp infected with original YHV strain. By single injection of dsRNA fragments, preparing based on non-coding sequence of either ORF1b or ORF3 regions from the YHV genome into 1g SPF Pacific whiteleg shrimp *Penaeus (Litopenaeus) vannamei*, then challenged with a 90% lethal dose of YHV-type 1 inoculums at the 2nd day later, we were able to demonstrate sequence-specific antiviral protection when compared with untreated control shrimp. At 14 days post-YHV challenge, survival rates of dsRNAs-treated shrimps were 67% with the ORF3- and 48% with the ORF1b-preparations. Although survivors were healthy, all were positive for YHV by reverse transcriptase polymerase chain reaction (RT-PCR) assay specific for the ORF1b sequence of YHV. Real-time PCR quantification of YHV copies prepared from gill tissues of RNAi-shrimps showed lower copy number compared with moribund shrimp. Sequencing analysis of ORF1b amplified from RNAi shrimp also showed similarity to YHV-type 1 without amino acid mutation or deletion. Positive immunohistochemical reactions for YHV structural protein gp116 in the lymphoid organs confirmed the presence of YHV (Fig. 1). On the other hand, lower immunofluorescent signal in the surviving shrimp than in the moribund control shrimp indicated not only lower viral loads in the former but also implied its active virulent. These results suggested that the surviving shrimps with dsRNA administration were tolerant rather than resistant to YHV infection. Consequence of RNAi could reduce YHV multiplication, but was not associated with absence or complete elimination of YHV.

Keywords: RNAi, virulent virus, yellow-head virus (YHV), yellow-head disease (YHD), penaeid shrimp, lethal dose, viral tolerant

Outputs

1. Gangnonngiw W, Anantasomboon G, Sang-oum W, Sriurairatana S, Sritunyalucksana K, Flegel T W. *Non-virulence of a recombinant shrimp nidovirus is associated with its non structural gene sequence and not a large structural gene deletion.* Virology **2009**; 385: 161-168.
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*Corresponding author.
Tel.: 0-2997-2222 ext. 1492; Fax: 0-2997-2222 ext. 1417
E-mail: ananta_rsu@yahoo.com

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P-C-72

Protection of Penaeid Shrimp from Mortality and Reduction of Yellow-head Virus (YHV) Multiplication Using Specific dsRNA Fragments Adminstration

Gun Anantasomboon [1*], Saengchan Saenapin.[2], Browdy CL.[3], Boonsirm Withyachumnarnkul.[2,4], Timothy W Flegel.[2]
[1] Anatomy unit, Department of Medical Sciences, Faculty of Science, Rangsit University, Patumthani, 12000.
[2] Center of Excellent for Shrimp Biology and Biotechnology (Centex Shrimp), Mahidol University, Thailand.
[3] South Carolina Department of Natural Resources and Holling's Marine Laboratory, Charleston, SC, USA.
[4] Department of Anatomy, Faculty of Science, Mahidol University, Rama 6 Rd. Bangkok 10400, Thailand.
*Corresponding author, e-mail: ananta_rsu@yahoo.com

Abstract

Yellow-head virus (YHV) is an invertebrate RNA virus in order *Nidovirales* that causes mass mortality called yellow-head disease (YHD) in penaeid shrimp. The original YHV (YHV-type1) outbreaks occurred in Thailand in the early 1990's. By injecting dsRNA preparations based on non-coding sequence of ORF1b and ORF3 regions of the YHV genome into Pacific whiteleg shrimp *Penaeus vannamei* challenged with a 90% lethal dose of YHV inoculum we were able to demonstrate sequence-specific antiviral protection when compared untreated control shrimp. At 15 days post-challenge the dsRNA treated shrimp survival was 67% with the ORF3 preparation and 48% with the ORF1b preparation compared to the control shrimp. Although survivors were grossly normal, all were positive for YHV-type1 by reverse transcriptase polymerase chain reaction (RT-PCR) assay specific for the ORF1b sequence. Real-time PCR quantification of YHV copies prepared from gill tissues of RNA interference (RNAi) in the surviving shrimps, as well as immunofluorescent labeling in lymphoid organ tissues confirmed lower YHV copy number compared to the moribund shrimps. Additional results of DNA sequencing analyses from parts of the ORF1b and the ORF3 regions indicated non-mutated aspect of the forming YHV population in shrimps. These results have suggested that the survival of YHV-infected shrimp is depended on the low viral load since RNAi could not completely inhibit YHV replication and was not associated with the production of defective YHV-type1 virion. They are likely tolerant rather than resistant to YHV-1 infection.

Keywords: RNAi, yellow-head virus, *Penaeus vannamei*, quantitative RT-PCR, DNA sequencing

Background

Yellow-head virus (YHV) is an invertebrate RNA virus in order *Nidovirales* that causes mass mortality called yellow-head disease (YHD) in penaeid shrimp. The original YHV outbreak had found in Thailand and Taiwan since the early 1990's. At present, 5 genetic variants are known in the YHV complex, but only the original YHV-type 1 variant appears to be highly virulent. Most studies have focused on acute type 1 viral infection (moribund shrimp), however, there is limited data on survivors of these outbreaks and the RNA interference (RNAi) technology that could protect shrimp from the YHD as well.

Objectives, Materials and Methods

The purposes of this study are to examine the percent survivors from a high dose (LD90) of virulent YHV-type1 challenge test combining with RNA interference (RNAi) procedure in 1g SPF Pacific whiteleg shrimps *Penaeus (Litopenaeus) vannamei*; to determine whether or not they were infected with YHV-1 and if so to determine some characteristics of the infection in terms of histopathology and immunohistochemical localization of the presented viruses under confocal laser scanning microscopy (CLSM); to quantify copy number of YHV replications in the survivors comparing with the moribund shrimps by real-time RT-PCR quantification; and to compare (ANOVA test) the parts of the ORF1b and ORF3 genomic sequences of the replicating YHVs, 27 and 26 inserted clones respectively, that were collected and amplified from the experimental shrimps.

Results

By injecting dsRNAs preparations based on ORF1b and ORF3 regions of the YHV genome, result demonstrate that non-coding dsRNAs designed from ORF1b and ORF3 regions induce sequence-specific antiviral protection when compared with untreated control shrimp. At 15 days post-challenge the dsRNA treated shrimp survival was 67% with the ORF3 (ORF3/YHV) preparation and 48% with the ORF1b

(ORF1b/YHV) preparation compared to low survival rate of 14% for the untreated control shrimp. Although survivors were grossly normal, but all were positive for YHV-type1 by reverse transcriptase polymerase chain reaction (RT-PCR) assay specific for the ORF3 sequence of YHV. The 739 bp amplicon from gill samples of surviving and moribund shrimp was amplified and demonstrated in agarose gel photograph. Positive immunohistochemical reactions for YHV structural protein, gp116 using V3-2B monoclonal antibody (Sithigorngul, 2000; 2002) in the lymphoid organ (LO) confirmed the presence of complete YHV virions. However, immunofluorescent signal revealed lower YHV load in the RNAi-surviving shrimps correlated to the lower copy number of YHV genome than that found in moribund and positive control shrimps by real-time RT-PCR quantifications (data not show). DNA sequencing with alignment analyses of the ORF1b (Fig. 1) and the ORF3 regions on the YHV genome show a few of single-base mutations, but there was no significant difference ($P<0.01$) among YHV population that were collected from the surviving and moribund shrimps.

Discussion and Conclusions

The results indicate that RNAi using dsRNAs administrations, prepared from non-coding sequences of ORF3 and ORF1b regions could protect shrimps from mortality with high dose (LD90) of YHV-type1 injection. It induces the specific antiviral immunity and reduces YHV multiplication in Penaeid shrimp. However, surviving shrimp from dsRNA treated groups were still positive YHV infection by quantitative RT-PCR and immunohistochemical analyses.

Somehow, the surviving phenomenal was correlated to low YHV copy number in the former and low amount of YHV-structural proteins, gp116, when compared to the moribund shrimp. In addition, surviving shrimp is not associated with mutated genome of the YHV. These results suggest that the surviving shrimp appear to be tolerant rather than resistant to YHV-1 infection. The mechanism that involves in limitation of YHV replication in the survivors is under investigated.

Acknowledgements

This study was funded by the Thailand Research Fund (TRF) for young scientism, grant no. MRG5180212 to Dr. Gun Anantasomboon. We would like to acknowledge the Department of Medical Sciences, Faculty of Science, Rangsit University and the Centex Shrimp, Mahidol University for providing of equipments and budgets.

References

1. Anantasomboon G, Poonkhum R, Sittidilokratna N, Flegel TW, Withyachumnarnkul B. Low viral loads and lymphoid organ spheroid are associated with yellow head virus (YHV) tolerance in white-leg shrimp *Penaeus vannamei*. Develop. Comp. Immunol. 2008; 32: 613-626.
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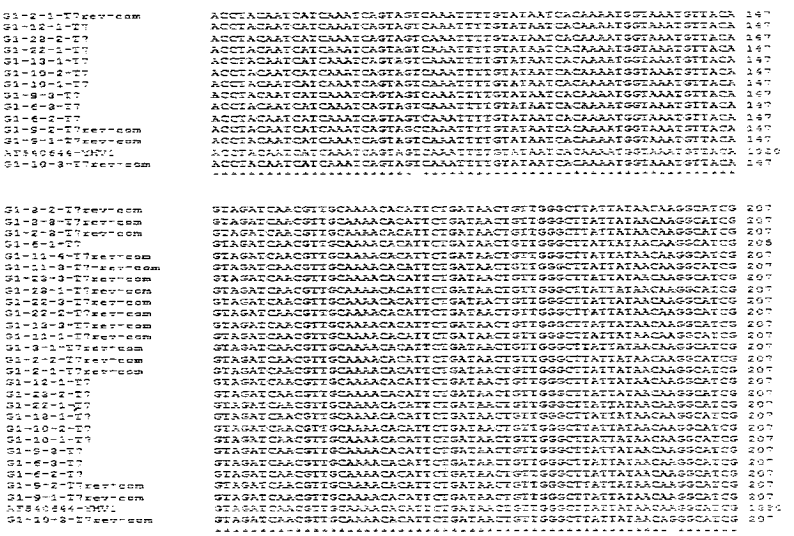

Fig.1 Alignment test of the ORF1b sequence from 27 collected clones showing a few single-base mutations without significant difference in genomic mutation ($P<0.01$) when compared to the original YHV-1. Almost of the replicated base sequences that were found in surviving shrimps are identity to the virulent YHV-type1 (purple line, and*).

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การประชุมวิชาการกายวิภาคศาสตร์แห่งประเทศไทย ครั้งที่ 35

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และ สมาคมกายวิภาคศาสตร์แห่งประเทศไทย

PP-24

Tolerant Property of Penaeid Shrimp Family to Virulent YHV is Correlated with Genomic Diversity: A Model Against Mortality of Invertebrates

Gun Anantasomboon[1*], Somneuk Nilbunga[2], Timothy W Flegel[3,4], Boonsirm Withyachumnarnkul[4,5]

[1] Anatomy unit, Department of Medical Science, Faculty of Science, Rangsit University, Patumthani 12000
[2] Department of Anatomy, Faculty of Medicine, Srinakharinwirot University, Bangkok 10110
[3] National Center for Genetic Engineering and Biotechnology (BIOTEC), NSTDA, Patumthani 12150
[4] Center of Excellent for Shrimp Biology and Biotechnology (Centex Shrimp); and [5] Department of Anatomy, Faculty of Science, Mahidol University, Bangkok 10400, Thailand

*Corresponding author, e-mail: ananta_rsu@yahoo.com

Abstract

Shrimp, as well as other invertebrates have no immune memory or adaptive immune response, therefore, there is no antibody reaction for viral and microbial defenses. Yellow-head virus (YHV) is a virulent RNA virus that causes mass mortality in penaeid shrimp, called yellow-head disease (YHD). The original YHV (YHV-type1) outbreaks occurred in Thailand in the early 1990's. At present, some YHV-type1 infected domesticated black tiger shrimp *Penaeus monodon* in commercial farms and wild broodstocks have no clinical signs and symptoms of YHD. It is believed that natural selection of survivors after YHV outbreaks has been occurred with Thai stocks. In this study, we examined the tolerant property of shrimp to YHD that is possibly correlated to the specific shrimp family. *P. monodon* families from stocks were immersive challenged with YHV-type1. At 30 days post-challenge, 8% of shrimps from one family were still survived. Microsatellites identification was subsequently inspected among the surviving and the moribund samples using their genomic DNA extracts. Result with CUPmo11 PCR-primer set revealed that all of the survivors contained unique microsatellites allele at 142 and 148 bps, which was different from the microsatellites scores of the moribund shrimp families. By TEM and RT-PCR examinations, all surviving shrimps were positive YHV infection. The results are able to demonstrate a model of a special *P. monodon* family with unique genomic diversity that is associated to the YHD tolerance. However, the specific genes and mechanism that are correlated to their tolerated property to the virus should be further investigated.

Keywords: Microsatellite markers, YHV, viral tolerance, *Penaeus monodon*, genomic diversity

Background

Microsatellites or Simple Sequence Repeats (SSRs) of animal is repetitive based-sequences, located within both of non-coding and coding regions of the genomic DNA. It is semi-conservatively passed from the parents to their offspring and is wildly applied as a source of genetic markers for identification of family or strain in invertebrates. Yellow-head virus (YHV) is an invertebrate ssRNA virus in order *Nidovirales* that causes mass mortality in penaeid shrimp cultivations, called yellow-head disease (YHD). The original YHV outbreak has been reported in Thailand and Taiwan since the early 1990's. At present, more than 5 genetic variants are known as the YHV complex virus, but only the original YHV-type 1 variant appears to be the most virulent. Previous studies have focused on acute YHV-1 infection, therefore, there are limiting data upon survivors after YHV outbreaks and remaining of unclear mechanism that are involved in the tolerance or resistance from mortality of the penaeid shrimp population.

Objectives

Purposes of this study are firstly to examine the percent survival of the black tiger shrimps *Penaeus monodon* from virulent YHV-type1 challenged test, and to determine whether or not there is genetic variance or genomic diversity among shrimp families that is involved in YHD-tolerated property.

Materials and Methods

Ten *P. monodon* families were obtained from stocks of Shrimp Genetic Improvement Center (SGIC), Surajthani province and a commercial farm in Chachungsao province. Ten shrimps from each batch of families with 10-15 g body weight were pooled-immersive challenged with YHV-type1 inoculums, and observed the number of survivors and moribund shrimps. During 30 days of experimental program, lymphoid organs, gills

and swimming legs of the moribund shrimps and the survivors were collected and subsequently prepared for TEM and RT-PCR examinations of YHV infection. Another inspection, their genomic DNAs were extracted and identified for their family stocks or genomic diversity. Based on microsatellite markers and PCR amplification, five sets of primers specifying for microsatellite markers of the *P. monodon* (Tassanakajon, 1998) were donated from Prof.Dr. Anchalee Tassanakajon at Chulalongorn University and were used in the microsatellites identification of shrimp family. Microsatellite-PCR products from the survivors and the moribund shrimps were finally visualized and differentiated by 1.5% polyacryramide gel electrophoresis.

Results, Discussion and Conclusions

By immersive challenge of virulent YHV-type1 with ten families of the *P. monodon* stocks, results demonstrated that 8 percent of the experimental shrimps still survived at the day 30. Interestingly, all of the survivors were originated from the same family by the result of microsatellites identification using CUPmo11 PCR-primer set. The result revealed a significant difference among the survivors and the moribund shrimps that all of the survivors have unique microsatellites allele at 142 and 148 bps (Fig.1), whereas, the moribund samples contain differential microsatellite scores as 139, 142, 148, 152, 157 bps of PCR products. The moribund shrimps were retrieved as other different families which are susceptible for YHV-type1 and have no tolerant property to the YHD. Although the survivors were healthy and grossly normal, but the results from TEM and RT-PCR examinations indicated all surviving shrimps (8 animals) were positive YHV infection (data not show). This suggests that the surviving shrimps appear to be tolerant rather than resistant to the YHV-type1 multiplication (Anantasomboon, 2008). In summary, the results are able to demonstrate a model of special *P. monodon* family with unique genomic diversity which is likely correlated to the YHD tolerance.

In addition, natural selection of the surviving shrimps that is occurred after disease outbreak has been purposed in the Pacific white-leg shrimp *Penaeus vannamei* against Taura syndrome virus (TSV). They have differences of genetic backgrounds, both of TSV-susceptible (Kona) and TSV-resistant strains. These properties can be passed to their offspring as unique families. Somehow, not only the survived phenomena is remained question, but also the mechanism in toleration to virulent virus of the shrimp should be further investigated.

Acknowledgements

This study was funded by the Thailand Research Fund (TRF) for young scientism, grant no. MRG5180212 to Dr. Gun Anantasomboon, Faculty of Science, Rangsit University. We would like to acknowledge Prof.Dr. Anchalee Tassanakajon, Faculty of Science, Chulalongkorn University for suggestions in genomic study of *P.monodon*; and the Centex Shrimp, Mahidol University for providing of equipments and additional budget.

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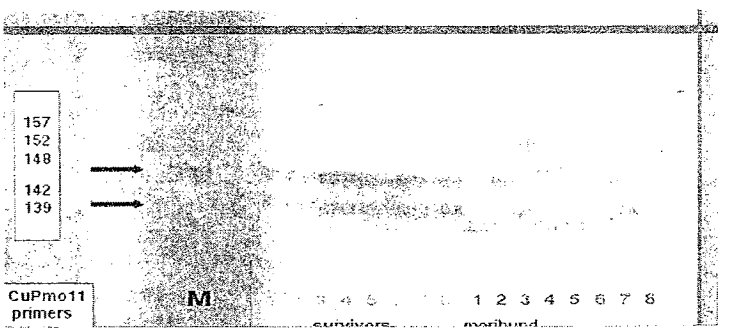

Fig.1 Gel electrophoresis exhibiting microsatellite inspection of survivors and moribund *P. monodon*. Unique bands of microsatellite alleles are found in the survivors as 142 and 148 bps. In contrast, the different patterns of microsatellite amplified bands (as 139,142,148, 152, 157 bps) are observed with the moribund samples.

TOLERANT PROPERTY OF PENAEID SHRIMP FAMILY TO VIRULENT YHV IS CORRELATED WITH GENOMIC DIVERSITY: A MODEL AGAINST MORTALITY OF INVERTEBRATES

Gun Anantasomboon[1,*], Somneuk Nilbu-nga[2], Timothy W Flegel[3,4] and Boonsirm Withyachumnarnkul[4,5]

[1]Anatomy Unit, Department of Medical Science, Faculty of Science, Rangsit University, Patumthani 12000;
[2]Department of Anatomy, Faculty of Medicine, Srinakharinwirot University, Bangkok 10110;
[3] National Center for Genetic Engineering and Biotechnology (BIOTEC), NSTDA , Ministry of Science and Technology, Patumthani 12120;
[4]Centex Shrimp; and [5]Department of Anatomy, Faculty of Science, Bangkok, 10400 Thailand

**Corresponding author, e-mail address:* ananta_rsu@yahoo.com

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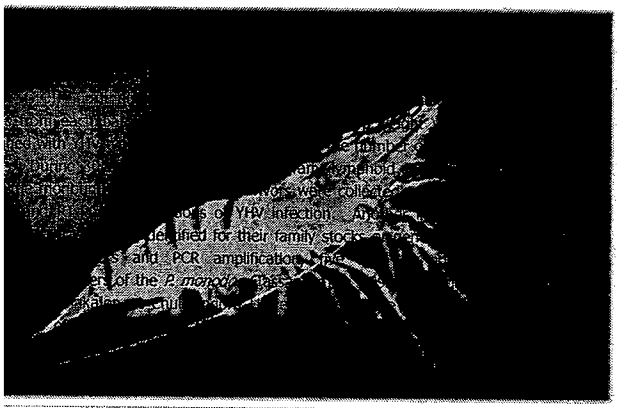

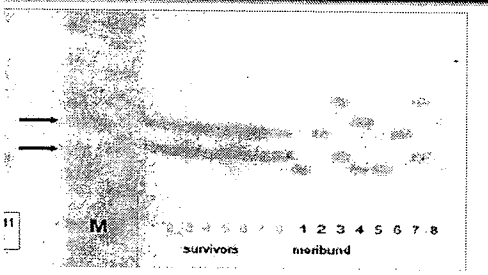

Polyacryramide gel electrophoresis with silver stain exhibiting microsatellite inspection of **survivors** and **moribund** *P. monodon*. Unique bands of microsatellite alleles are found in the survivors as 142 and 148 bps. In contrast, the different patterns of microsatellite amplified bands (as 139,142,148, 152, 157 bps) are observed with the moribund samples.

Introduction

Microsatellites or Simple Sequence Repeats (SSRs) of animal is repetitive based-sequences, located within both of non-coding and coding regions of the genomic DNA. It is semi-conservatively passed from the parents to their offspring and is wildly applied as a source of genetic markers for identification of family or strain in invertebrates. Yellow head virus (YHV) is an invertebrate ssRNA virus in order *Nidovirales* that causes mass mortality in penaeid shrimp cultivations, called yellow-head disease (YHD). The original YHV outbreak has been reported in Thailand and Taiwan since the early 1990's. At present, more than 5 genetic variants are known as the YHV complex virus, but only the original YHV-type 1 variant appears to be the most virulent. Previous studies have focused on acute YHV-1 infection, therefore, there are limiting data upon survivors after YHV outbreaks and remaining of unclear mechanism that are involved in the tolerance or resistance from mortality of the penaeid shrimp population.

Conclusions and Discussion

In summary, the results are able to demonstrate a model of special *P. monodon* family with unique genomic diversity which is likely correlated to the YHD tolerance. In addition, natural selection of the surviving shrimps that is occurred after disease outbreak has been purposed in the Pacific white-leg shrimp *Penaeus vannamei* against Taura syndrome virus (TSV). They have differences of genetic backgrounds, both of TSV-susceptible (Kona) and TSV-resistant strains. These properties can be passed to their offspring as unique families. Somehow, not only the survived phenomena is remained question, but also the mechanism in toleration to virulent virus of the shrimp should be further investigated.

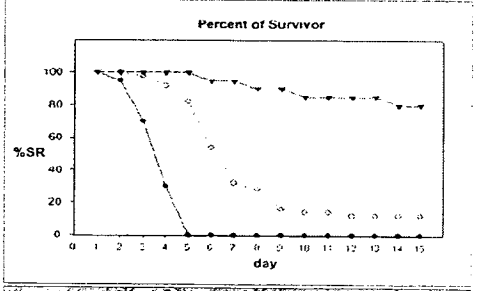

Figure 1. Graph showing percent survival of 10 *P. monodon* families that were immersive-challenged with virulent YHV (YHV-type1; o). After bioassay program, survival rate (%SR) of co-habitated shrimps (o) at 15 to 30 days was 8%, n = 8/100). Other groups of the positive control shrimps with YHV injection (•) were absolute die = 0%, survivor, n = 0/20) within 5 days, post YHV injection. Whereas, the shrimp from negative control group (▼) had remained at 80.0% (n = 16/20).

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/ immersive challenge of virulent YHV-type1 with ten families of the *P. monodon* stocks, results demonstrated ercent of the experimental shrimps still survived at the day 15 to 30 (Fig.1; o). Interestingly, all of the survivors als) were originated from the same family by the result of microsatellites identification using CUPmo11 PCR et. The result revealed a significant difference among the survivors and the moribund shrimps that all of the s have unique microsatellites allele at 142 and 148 bps (Fig.2), whereas, the moribund samples contain al microsatellite scores as 139, 142, 148, 152, 157 bps of PCR products. The moribund shrimps were retrieved different families which are susceptible for YHV-type1 and have no tolerant property to the YHD. Although the s were healthy and grossly normal, but the results from RT-PCR and TEM examinations indicated all surviving were positive YHV infection (Fig.3&4). This suggests that the surviving shrimps appear to be tolerant rather istant to the YHV-type1 multiplication (Anantasomboon, 2008).

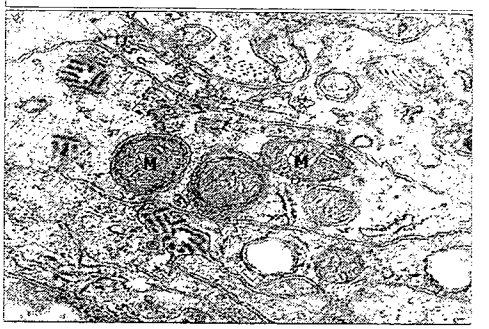

Figure 4. Transmission Electron Micrograph (TEM) showing YHV particles (*), depositing in cytoplasm of cells in the lymphoid organ of the survived shrimp; M = mitochondria

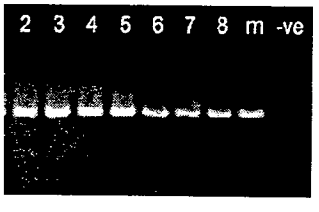

Figure 3. Gel electrophoresis stained with ethidium bromide showing positive RT-PCR results of RNA extracts from gills of surviving and control shrimps from YHV-type1 challenged test. The primer set for 739 amplicon of the YHV was designed from ORF3 region, and used for RT-PCR analysis. Lane M, 100 bp DNA marker; Lanes 1-8, surviving shrimps at day 30; Lane m, moribund shrimp at day 5; Lane -ve, negative control shrimp. Note that all of the survivors are positive YHV-infection.

Acknowledgements

This study was funded by the Thailand Research Fund (TRF) for young scientism, grant no. MRG5180212 to Dr. Gun Anantasomboon, Faculty of Science, Rangsit University. We would like to acknowledge Prof.Dr. Anchalee Tassanakajon, Faculty of Science, Chulalongkorn University for suggestions in genomic study of *P. monodon*, and the Centex Shrimp, Mahidol University for providing of equipments and additional budget.

INJECTION OF NON-CODING dsRNA SPECIFIC TO YELLOW-HEAD VIRUS (YHV) GENOME INDUCES PROTECTION OF PENAEID SHRIMP FROM MORTALITY AND REDUCEDS YHV MULTIPLICATION

Gun Anantasomboon*, Sangchan Saenapin, Craig L Browdy, Boonsirm Withyachumnarnkul and Timothy W Flegel

Anatomy Unit, Department of Medical Science
Faculty of Science
Rangsit University
Pathum-thani 12000, Thailand
ananta_gun@yahoo.com

Yellow-head virus (YHV) is an invertebrate RNA virus in order *Nidovirales* that causes mass mortality called yellow-head disease (YHD) in penaeid shrimp. The original YHV outbreak had found in Thailand and Taiwan since 1990. At present, several types of YHV family are discovered in many Asian countries, but only the original YHV-type 1 appears to be virulent.

Here we compared 1g surviving and moribund Pacific white-leg shrimp *Penaeus (Litopenaeus) vannamei* that were challenged with 90% lethal dose of YHV inoculum, combining with dsRNA administration. Result show that non-coding dsRNAs designed from ORF1b and ORF3 regions of YHV genome induces sequence-specific antiviral protection comparing to the low survival rate of control shrimp (Fig. 1). Fourteen days after RNA interference (RNAi), the experimental shrimp revealed survival rates at the level of 67% (ORF3/ YHV) and 48% (ORF1b/ YHV). Although survivors were grossly normal, but all were positive for YHV-type 1 by reverse transcriptase polymerase chain reaction (RT-PCR) assay specific for the ORF1b sequence of YHV. Positive reaction of immunohistochemical analysis of YHV structural protein, gp116 in lymphoid organ and gill tissues has confirmed that the YHV virions and its envelope protein were completely produced. However, immunofluorescent signal was differential lower in the surviving shrimp. Results also suggest that the surviving shrimp appear to be tolerant, but not resistant to YHV-1 infection and RNAi was not associated with absence of the YHV. Instead, it was associated with the presence of YHV-positive and likely relative to low viral replication in the survivors.

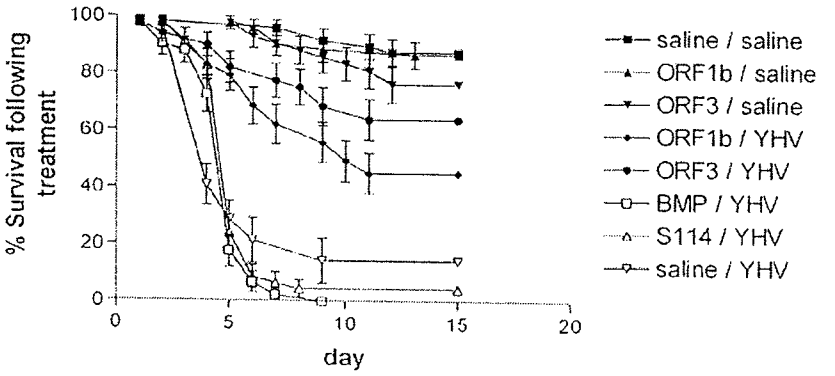

FIGURE 1. Graph showing percent survival of RNAi shrimp. At day 15, *Penaeus vannamei* that were injected with dsRNAs specific to non-coding sequences of YHV-ORF1b and -ORF3 combining with high lethal dose (LD90) of YHV inoculum reveal survival rates at 48% (ORF1b/ YHV) and 67% (ORF3/ YHV) respectively.

INJECTION OF dsRNA SPECIFIC TO NON-CODING SEQUENCES OF YELLOW-HEAD VIRUS (YHV) GENOME INDUCES PROTECTION OF PENAEID SHRIMP FROM MORTALITY AND REDUCES YHV MULTIPLICATION

Gun Anantasomboon[1,2*], Saengchan Senapin[2,3], Craig L Browdy[4], Boonsirm Withyachumnarnkul[2] and Timothy W Flegel[2]

[1]Anatomy Unit, Department of Medical Science, Faculty of Science, Rangsit University, Patumthani 12000;
[2]Centex Shrimp and Department of Anatomy, Faculty of Science, Mahidol University, Bangkok 10400;
[3]BIOTEC, Ministry of Science and Technology, Patumthani 12120; Thailand
[4]South Carolina Department of Natural Resources (SCDNR) and Hollings Marine Laboratory, SC, 29412 USA.
***e-mail address:** ananta_gun@yahoo.com*

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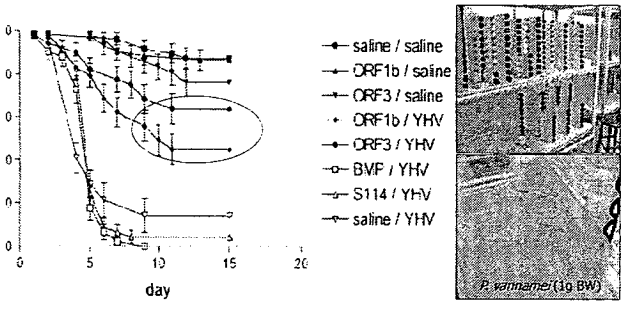
erials, Methods and Results

we compared 1g surviving and moribund Pacific white-leg shrimp *Penaeus penaeus) vannamei* that were injected with dsRNA into 3rd-4th abdominal muscle, lining with 90% lethal dose (LD90) of YHV inoculum at day 2 later. By injecting As preparations based on ORF1b and ORF3 regions of the YHV genome, result onstrate that non-coding dsRNAs designed from ORF1b and ORF3 regions induce ence-specific antiviral protection when compared with untreated control shrimp 1). At 15 days post-challenge the dsRNA treated shrimp survival was 67% with RF3 (ORF3/YHV) preparation and 48% with the ORF1b (ORF1b/YHV) preparation ared to low survival rate of 14% for the untreated control shrimp. Although vors were grossly normal, but all were positive for YHV-type1 by reverse criptase polymerase chain reaction (RT-PCR) assay specific for the ORF3 ence of YHV (Fig. 2). The 739 bp amplicon from gill samples of surviving and ound shrimp was amplified and demonstrated in agarose gel photograph. ve immunohistochemical reactions for YHV structural protein, gp116 using V3-2B nclonal antibody (Sithigorngul, 2000; 2002) in the lymphoid organ (LO) confirmed resence of YHV. However, the immunofluorescent signal was lower in the RNAi-ving shrimps correlated to lower copy number of YHV genome than in moribund ol shrimp by real-time PCR quantification (data not show).



e 1. Graph showing percent survival of shrimp treated or not with dsRNAs. At day 15 post-administration with dsRNAs and an LD90 dose of YHV later, SPF *Penaeus vannamei* injected with dsRNA specific to sequences of YHV-ORF1b and –ORF3 showed significant levels of protection (48% survival with ORF1b/YHV (♦) dsRNA and 67% survival with ORF3/YHV (●) dsRNA) against mortality from yellow-head disease compared to result from other control groups: saline/saline injection (■); ORF1b dsRNA/negative YHV (▲); ORF3 dsRNA/negative YHV (▼); non-shrimp dsRNA(BMP)/YHV (□); catfish dsRNA(S114)/YHV (Δ); and saline/positive YHV(▽).

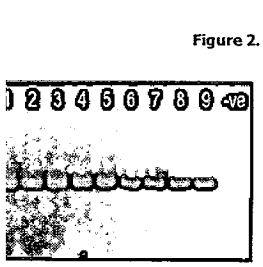


Figure 2. Ethidium bromide stained electrophoresis gel showing positive semi-quantitative RT-PCR results of RNA extracts from gills of control shrimp and shrimp from dsRNAs treated groups. The primers for 739 amplicon was designed from ORF3 region of the YHV and used for RT-PCR analysis at 35 cycles. Lane M, 100 bp DNA standard marker; Lanes 1-5, example of moribund shrimp at day 2 from positive YHV (▽); Lanes 6-9, surviving shrimp from dsRNA treated groups: 6 (♦), 7 (●), 8 (□), 9 (Δ); Lane -ve, negative control shrimp (■) result at d5. Note that all of the bands for survivors are less intense than those of moribund shrimp.

Objectives

The purpose of this study is to examine survivors of a YHV-type 1 challenge test combining with RNA interference (RNAi) to YHV infection in shrimp and to determine whether or not they were infected with YHV-1 and if so to determine some characteristics of the infection in terms of survival rate, histopathology, copy number of YHV replications and immunohistochemical characteristics of the virus present.

Conclusions and Discussion

The results indicate that RNAi using dsRNAs prepared from non-coding sequences from ORF3 and ORF1b regions could protect shrimp from mortality at high dose (LD90) of YHV injection and reduces YHV multiplication. However, surviving shrimp from dsRNA treated groups were positive YHV-type 1 infection by semi-quantitative RT-PCR analysis. Furthermore, surviving shrimp was related to low YHV copy number in the former and low amount of YHV-structural proteins, gp116, when compared to the moribund shrimp by real-time PCR and immunofluorescent detection. These results suggest that the surviving shrimp appear to be tolerant rather than resistant to YHV-1 infection since RNAi was not associated with absence of YHV. It is also hypothesized that RNAi generated by the dsRNAs tested partially blocked the synthesis of viral proteins which may have showed but not eliminated viral replication.

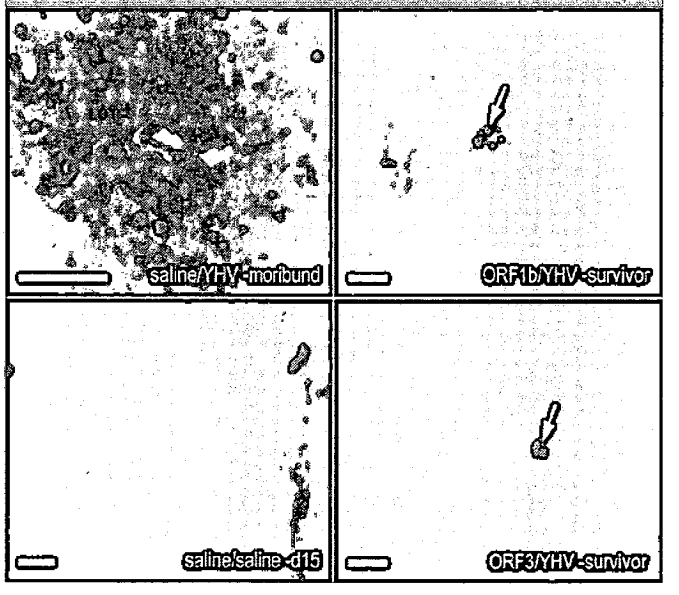


Figure 3. Photomicrographs showing immunofluorescent labeling for structural protein, gp116 of YHV in the lymphoid organ (LO) sections from experimental *P. vannamei*. Immunofluorescent signals (yellow-green color) of moribund shrimp at day 2 after YHV injection (saline/YHV) are intensely observed in the LO tubular (LOT) matrices. Whereas, immuno-reactions in surviving shrimp treated with dsRNAs (ORF1b/YHV & ORF3/YHV) reveal mildly positive signal with some infected cells at clusters of aggregated hemocytes (arrows), but are very faint to negative in the LOT. Bar = 50 μm.

Acknowledgements

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