INFECTIOUS MYNECROSIS VIRUS (IMNV): AN EMERGING PATHOGEN FOR SHRIMP FARMING IN INDONESIA

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Part One

Current Status of *Infectious Myonecrosis Virus* (IMNV) in Pacific White Shrimp (*Litopenaeus vannamei)* in Indonesia

Introduction

Indonesian shrimp production at 352.855 tonnes (Directorate General of Aquaculture, 2010) is valued in excess of $2 billions/year. Undoubtedly, the shrimp industry is an important natural resource for Indonesia, a pivotal role on rural development and a significant export industry. Due to the decrease of the production of black tiger shrimp (*Penaeus monodon*), Pacific white shrimp (*Litopenaeus vannamei*) was illegally introduced to Indonesia in 1999. The exotic shrimp was officially introduced for research and culture purposes in 2000 and 2001, respectively (Ministerial Decree No. 4/2001). Since then *L. vannamei* aquaculture dominated Indonesian shrimp aquaculture and contributed about 60% of Indonesian shrimp production. By the end of 2007, *L. vannamei* has been cultured in at least 17 provinces including East Java, Central Java, Yogyakarta, West Java, Banten, Lampung, South Sumatera, Riau, Bengkulu, West and North Sumatera, West Kalimantan, South Kalimantan, East Kalimantan, Bali, West Nusa Tenggara and South Sulawesi. Clearly, Pacific white shrimp is the most popularly cultivated shrimp species in intensive shrimp culture systems in Indonesia. The non-native shrimp is thought to be resistance to white spot syndrome virus (WSSV) and capable of producing high yield. With stocking density of 200 PLs/m^2, shrimp farmer could produce 50-70 MT/ha/crop, at least before disease outbreaks hit cultured *L. vannamei*.

Two years following the introduction of the exotic shrimp to Indonesia, the shrimp industry was hit by disease outbreaks caused by Taura syndrome virus (TSV). The first disease outbreak associated with TSV in *L. vannamei* was reported in East Java in 2003. The spread of the disease and economic losses due to the outbreaks are considered to be limited as farmer used PLs of specific pathogen resistance to TSV. Another disease, infectious hematopoietic and hypodermal necrosis virus (IHHNV), was also reported to cause slow growth and runt deformity syndrome in farmed Pacific white shrimp in Indonesia. Although the mortality due to the virus is quite low, the disease significantly decrease total yield of affected farms.

New emerging disease

In May 2006, a mass mortality of cultured *L. vannamei* was reported in district of Situbondo, East Java (Nur'aini *et al*., 2006). The affected shrimp showed clinical signs of whitish necrotic muscle and reddened tail fan, similar to those reported for infectious myonecrosis virus (IMNV) disease in Brazil (Ligthner *et al*., 2004a; 2004b). IMNV is a non-enveloped, double-stranded RNA virus classified to the family of *Totiviridae* (Poulos *et al*., 2006). The results of histopathological examination and PCR test of diseased shrimp confirmed that the outbreak was associated with IMNV infection (Nur'aini *et al*., 2006; Taukhid and Nur'aini, 2008). Subsequent analysis of complete genome sequences revealed that the Indonesian IMNV shares 99.6% identity to that of Brazilian IMNV (Senapin *et al*., 2007). The high identity of the genome sequences of IMNV from Brazil and Indonesia indicates that Indonesian IMNV may be originated from Brazil. This is another classic example of trans-boundary disease which occurred due to irresponsible movement of contaminated stocks for aquaculture (Flegel, 2006).

Host range and clinical signs

Although *P. monodon* and *P. stylirostris* are susceptible to experimental infection with IMNV (Tang *et al*., 2005) and both species are being cultured in Indonesia, morbidity and mortality associated with IMNV are restricted to *L. vannamei*. The affected shrimp showed lethargy, swimming on the water surface and near the dyke of the pond at night time, whitish necrotic muscle and reddened, particularly in the tail fan. The disease and mortality usually, but not always, occurred in shrimp at 30-90 days of culture. Following the presence of clinical signs in affected shrimp, the feeding rate decreased and daily mortality of shrimp increased. Losses due to mortality rate range from 40 to 60%.

Diagnosis

Diagnostic methods for IMNV was established based on standard of FAO/NACA/OIE (Bondad-Reantaso *et al*, 2001) i.e. level I, II and III diagnosis. Level I diagnosis was based on environmental observation and clinical signs of diseased shrimp. The appearance of whitish necrotic muscle and reddened tail fan in diseased shrimp was used as a presumptive diagnosis of

IMNV in farm-level. Level II diagnosis was based on histopathological changes in infected shrimp. Histopathological changes of diseased shrimp including coagulative necrosis of striated muscle (myonecrosis), haemocytic infiltration and fibrosis (Taukhid and Nur'aini, 2008) was used as a tentative diagnosis for IMNV. Level III diagnosis based on molecular biological data was used for confirmative diagnosis. RT-PCR detection of KHV was carried out using commercially available diagnostic kits (IQ 2000, Taiwan) and specific primers set developed by Serapin *et al*. (2007).

Geographical distribution

Since the first outbreak of IMNV in 2006 in East Java, one of central production of shrimp in Indonesia, and South Kalimantan, the disease has spread to other provinces where *L. vannamei* is being cultured (Nur'aini *et al*., 2006). By the end of 2007, the outbreak spread to the east (Bali and West Nusa Tenggara provinces) and to the west (Lampung province). In 2008, outbreaks associated with IMNV were reported in West Java and Riau provinces. By 2009, the disease was detected in North Sumatera, West Kalimantan, and South Sulawesi (Figure 1).

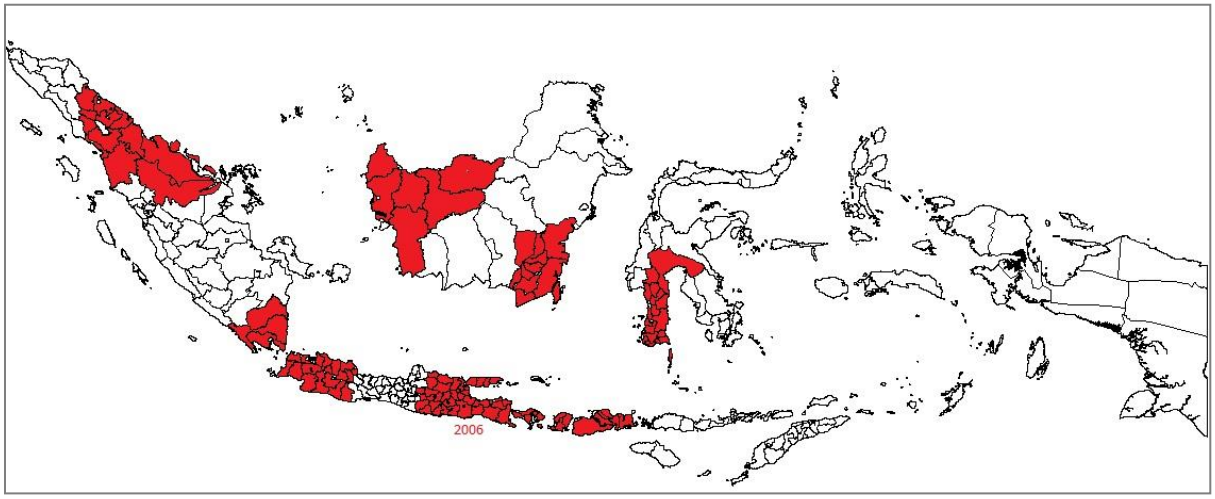

Figure 1. Geographical distribution of IMNV in Indonesia. Red colour indicates IMNV-infected provinces. Note the first outbreak of IMNV in East Java Province in 2006.

Current threat

Pacific white shrimp is not only commonly cultivated in Indonesia, but also being cultured in many Asian countries including Malaysia, the Philippines, Thailand, Vietnam and China, among others. To date, IMNV has only been reported to occur in Brazil and Indonesia. However, due to rapid spread of the outbreak to other provinces in Indonesia and large scale movement of the

shrimp stocks across the region and the globe, there is a high potential risk of spreading the disease to neighboring *L. vannamei*-culturing countries. Public awareness campaign, preparedness on IMNV and response to potential disease outbreak are needed in these countries.

Economic losses

Although disease outbreaks associated with IMNV has occurred in Indonesia for five years and losses due to mortality range from 40-60%, there is no official data available for economic losses. However, based on the decreased of *L. vannamei* production from 208,648 MT in 2008 to 170,699 MT (2009) and 157,525 MT (2010), it is estimated that IMNV caused direct economic losses of $200 millions over two years (2009 to 2010). The indirect economic losses are even bigger from those figures. For example, as IMNV kills the shrimp slowly, farmers believe that they can maintain the crop, and therefore, keep feeding the shrimp. As a result, feed conversion ratio (FCR) of infected population increase from normal value of 1.5 to 4 or higher, increasing the production cost. The decreased of production mainly due to low survival rate of cultured *L. vannamei*. For instance, in Lampung province, one of central production of farmed *L. vannamei*, the survival rate of cultured Pacific white shrimp has decreased about 27% over four years i.e. 82% (2007), 72% (2008), 64% (2009) and 55% (2010).

Prevention and control

Regulation. Fish Quarantine Office has quickly added IMNV to the List of Quarantine Fish Diseases through the Ministerial Decree No. 17/2006. The degree was aimed to protect the country from the incursion of exotic pathogens including IMNV and to prevent further spread of the diseases to uninfected areas. Unfortunately, this regulation is not well supported with contingency plan or technical implementation plan of dealing with outbreak of new emerging diseases (Sunarto and Cameron, 2005). Implementation of responsible movement of life shrimp is facing a significant challenge as IMNV is a new emerging disease, and therefore, lack of human resources, laboratorium capability, and resource expert group.

Prevention. Good aquaculture practices and application of biosecurity principles have been proved to be the most reasonable approach to prevent disease outbreaks including one caused by

IMNV. The application of biosecurity includes proper pond preparation, sterilization of intake water and equipments, and the use of SPF seed.

Control. Although there are no validated control measures for IMNV, scattered reports from farmers indicate that, during the early stage or low level of infection, improved water quality parameter and partial harvest of shrimp may reduce the mortality rate due to the outbreak. Usually, partial harvests were done 4 times per crop. For example, if a pond was stocked with 150 PLs/m^2, the partial harvests were done when the average body weight of shrimp is about 10 g, 15 g, 20 g and 25 g. However, in severely infected farm, immediate harvest must be done to prevent further losses, particularly if the shrimp has reached marketable size.

Research Needs

To date, IMNV has only been reported in Brazil and Indonesia, and therefore, there is limited information available regarding:

1. Epidemiology of the disease: how the virus transmits and spreads, how long the virus survive in the water, is there any carriers for the virus?
2. Farmers reported that many of IMNV outbreaks occurred following environmental changes such as raining, fluctuation of oxygen and pH, increased total Vibrio, and plankton blooms, particularly by blue green algae. What are factors triggering of IMNV outbreak? Is stress such as depleted water quality parameters is the triggering factors? If is so, what are the key and supporting parameters of water quality that trigger the outbreak?
3. Early warning systems: are there any sensitive and reliable detection tools for IMNV that allow quantification viral load and grading the severity of the disease (low, moderate & severe infections)? These tools may help farmers to make a better decision on whether they should continue the culture or do immediate harvest.
4. Most of shrimp farmers currently use certified IMNV-free PLs. However, outbreaks of IMNV occurred in isolated farms (hundreds kilometre away from other farms) which were stocked with specific pathogen free (SPF) PLs. Are PCR-based detection tools sensitive enough to detect low level of IMNV infection in PLs?

5. As IMNV has spread to major areas-culturing *L. vannamei* in Indonesia, the used of PL of specific pathogen resistant (SPR) to IMNV may be a better approach of controlling the disease in the country.

References

Bondad-Reantaso M.G., S.E. McGladdery, I. East and R.P. Subasinghe (eds.). 2001: Asia Diagnostic Guide to Aquatic Animal Diseases. FAO Fisheries Technical Publication Paper No. 402, Supplement 2, FAO, Rome, 240 pp.

Flegel, T.W. 2006. The special danger of viral pathogens in shrimp translocated for aquaculture. Sci. Asia 32, 215–231.

Lightner, D. V., C. R. Pantoja, B.T. Poulos, K.T.F. Tang, R.M. Redman, T. Andreas and J. R. Bonami. 2004a. Infectious myonecrosis (IMN): a new virus disease of *Litopenaeus vannamei*. In Aquaculture 2004 Book of Abstracts, p. 353. Baton Rouge, LA: World Aquaculture Society.

Lightner, D. V., C. R. Pantoja, B.T. Poulos, K.T.F. Tang, R.M. Redman, T. Pasos de Andrade and J. R. Bonami. 2004b. Infectious myonecrosis: new disease in Pacific white shrimp. Glob Aquac Advocate 7, 85.

Nur'aini, Y. L., B. Hanggono, S. Subyakto and Triastutik, G. 2006. Active surveillance of infectious myonecrosis virus (IMNV) in Pacific white shrimp (*Litopenaeus vannamei*) in East Java and Bali. Jurnal Perikanan (*J. Fish. Sci*.) IX (1): 1 – 8. In Indonesian

Senapin, S., K. Phewsaiya, M. Briggs and T.W. Flegel. 2007. Outbreaks of myonecrosis virus (IMNV) in Indonesia confirmed by genome sequencing and use of an alternative RT-PCR detection method. Aquaculture 266: 32-38.

Sunarto A. and A. Cameron. 2005. Response to mass mortality of carp: an Indonesian experience. In: Regional Workshop on Preparedness and Response to Aquatic Animal

Health Emergencies in Asia (ed. by R.P. Subasinghe & J.R. Arthur). FAO Fisheries Proceedings 4, 87–106.

Tang K. F., C.R. Pantoja, B.T. Poulos, R.M. Redman and D.V. Lightner. 2005. In situ hybridization demonstrates that *Litopenaeus vannamei*, *L. stylirostris* and *Penaeus monodon* are susceptible to experimental infection with infectious myonecrosis virus (IMNV). Abstract. Dis. Aquat. Org. 63: 261 – 265.

Taukhid and Y.L. Nur'aini. 2008. Infectious myonecrosis virus (IMNV) in Pacific white shrimp, Litopenaeus vannamei, in Indonesia. Indonesian Aquaculture Journal, 3: 139-146.

Part Two

At Low Temperature, Indonesian and Brazilian Infectious Myonecrosis Virus (IMNV) Isolates Show Different Infections in Shrimp (*Penaeus vannamei*)

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Infectious myonecrosis virus (IMNV) infects the Pacific white shrimp (*Penaeus vannamei*) and belongs to the *Totiviridae* family. IMNV was reported for the first time in Brazil in 2003/2004, and in Indonesia in 2006. The virus can cause up to 70% mortalities in the infected population. Different challenge studies were conducted to test the effects of temperature on IMNV infections in shrimp via intramuscular injection, over a period of 21 days. The first study was conducted in Indonesia using the Indonesian IMNV isolate at 26°C, 30°C, and 34°C, with final survivals of 93.3%, 0%, and 86.7%. These findings suggest that 30°C was the optimum temperature for IMNV infection, and reduced mortalities in shrimp in both 26°C and 34°C. To confirm if temperatures had similar effects on the Brazilian IMNV isolate, a second challenge study was conducted at the University of Arizona at 26°C and 30°C, with final survivals of 13.8% and 18.5%. As the effects of temperature on both isolates were different, a third challenge experiment with smaller number of animals was conducted to compare the Indonesian and Brazilian isolates at 26°C and 30°C. The final survivals for the Indonesian IMNV were 100% and 20%, while for the Brazilian isolate groups were 20% and 10%. The findings from the first and third experiment confirmed that the Indonesian IMNV isolate was optimum at 30°C, and low or no mortalities at 26°C. The findings from the second and third studies showed that the Brazilian isolate caused high mortalities in both temperatures. The different effects of low temperature on the Indonesian and the Brazilian isolates need further investigations. Clinical signs, histopathology, and RT-PCR analysis confirmed the presence of IMNV in the dead shrimp and survivors.

Keywords: IMNV, temperature, Indonesia, Brazil, *P. vannamei*

1. Introduction

1.1. Overview of the Totiviridae family

Infectious myonecrosis virus (IMNV) infects the Pacific white shrimp (*Penaeus vannamei*) and belongs to the *Totiviridae* family (Poulos et al., 2006; Nibert, 2007; Tang et al., 2008) in an unassigned genus. *Totiviridae* includes a number of viruses that are icosahedral virions, ranging between 30 and 40 nm in diameter, and with monosegmented double-stranded RNA (dsRNA) genomes. The genomes have overlapping open reading frames encoding a capsid protein (CP) and an RNA-dependent RNA polymerase/RdRp (Liu et al., 2010).

The members of the *Totiviridae* family mostly infect fungi and a number of medically important protozoan parasites such as Leishmania and Giardia (Goodman, et al. 2011). IMNV in the Pacific white shrimp was thought to be a unique member of the *Totiviridae*, or might represent a new dsRNA virus family that infects invertebrate hosts (Poulos and Lightner, 2006). Later, *Drosophila* totivirus (DTV), a member of *Totiviridae* family were also found infected fruit fly *Drosophila* (Wu et al., 2010), two mosquitoes *Armigeres* sp were infected by Armigeres subalbatus totivirus (AsTV), and *Culex* sp. by Omono River virus/OMRV (Zhai et al., 2010; Isawa, 2011). Haugland et al. (2011) identified that the Atlantic Salmon (*Salmo solar*) was infected by a piscine myocarditis virus (PMCV) belongs to the family. Both IMNV and PMCV cause myonecrosis, while IMNV causes skeletal muscle necrosis of shrimp, PMCV infection results in cardiomyocyte necrosis and inflammation. In plant hosts, the closest viruses would be the Partitiviridae family which includes the black raspberry F virus (GenBank accession number NC_009890), the southern tomato virus (STV) and the blueberry latent virus (BBLV) described by Sabanadzovic et al. (2009) and Martin et al. (2011). Based on genome organization with overlapped region in the CP and RdRp, both plant viruses share similar characteristic to *Totiviridae* members. Therefore, they are considered as a link or amalgam between *Totiviridae* and *Partitiviridae*.

From evolution and pathogenicity point of view, RNA viruses, due to their high mutation rate exist as a quasi-species. Under certain prevailing genetic and environmental pressure, novel strain arises in the RNA population that causes severe disease. This process is well known for many RNA viruses. Therefore, the identification of the key factors for virulence is critical in

determining the effect of mutations on pathogenicity. Liu (2010) suggested that horizontal transfer of dsRNA viral genes is widespread among eukaryotes and may give rise to functionally important new genes. The finding has consequences that RNA viruses may play significant roles in the evolution of eukaryotes.

1.2. Overview of IMNV

Infectious myonecrosis virus (IMNV) infecting cultured shrimp (*P. vannamei*), was reported for the first time in Brazil during 2002/2003, and was identified at the University of Arizona based on histopathology findings (Lightner et al., 2004) and viral purification (Poulos, et al., 2006). Even though the clinical signs were already seen in Indonesia during 2004/2005, the disease was not confirmed in the country until 2006 and was immediately reported by the National Coordinator to the Network of Aquaculture Centers in Asia-Pacific/NACA. Subsequent analysis revealed that the Indonesian IMNV sample had 99.6% nucleic acid sequence identity to that of Brazilian IMNV (Senapin et al., 2007). Because of the ever increasing importance of shrimp culture in the Asia-Pacific and the large scale boundary movement and culture of the species, IMNV was considered important for the region by the UN-FAO and the World Animal Health Organization (OIE) in 2006. In 2010, the Indonesian Department of Fishery Affairs listed IMNV as the most significant shrimp disease. There is no official report for IMNV in countries other than Brazil and Indonesia. With a relatively small number of samples from IMNV suspected cases selected by farmers and technical consultants, Senapin et al. (2011) concluded that IMNV has not spread to other suspected countries in Asia (China, Taiwan, India, Malaysia, Thailand, and Vietnam).

Both in Brazil and Indonesia, the natural infections that caused morbidity and mortality were found in *P. vannamei*. Based on experimental infections, the Giant tiger shrimp (*Penaeus monodon*), the Pacific blue shrimp (*P. stylirostris*), and the wild Southern brown shrimp (*P. subtilis*) were susceptible to IMNV (Tang et al., 2005; Coelho et al., 2009). Direct interview with farmers described that in Indonesia, shrimp are more susceptible to IMNV between 30 - 90 days of culture. During 2005-2007, most of the shrimp farms in Indonesia experienced IMNV after 90 days. This became 60 days in 2009/2010, and recently the disease infects the shrimp at 30 days.

IMNV causes a slowly progressive disease with cumulative mortalities of up to 70% in acute stage or 40-50% in the chronic stage. The affected shrimp showed clinical signs of whitish necrotic muscle and reddened tail fan (Lightner et al., 2004). Histopathology analysis shows severe necrosis of the skeletal muscle and lymphoid organ spheroids. The disease is currently diagnosed using a combination of gross signs, histopathology, and in situ hybridization with a gene probe. A rapid and sensitive method was developed using reverse-transcriptase polymerase chain reaction/ RT-PCR (Poulos & Lightner, 2006).

1.3. Effects of temperature on shrimp viral diseases

Environmental parameters such as salinity and temperature have have consequences for both the host and the pathogen involved. Water temperature affects aquatic host health by directly influencing the metabolism, oxygen consumption, molt cycle, and growth (Allan et al., 2006). When considering shrimp defense mechanisms and optimum temperature, *P. vannamei* of less than 5 gram grow better at temperature higher than 30°C. For shrimp larger than 16 gram, the optimum temperature is around 27°C (Wyban et al., 1995).

For the viruses, temperature is known to influence several steps in the viral replication process, for example in the infectious pancreatic necrosis virus (Roberts and Dobos, 1983), where the virus was optimum at 20°C and failed to replicate at 28°C, neither virus-specific mRNA nor virus-specific polypeptides could be detected. Shifting the viruses from permissive temperature (20°C) to non-permissive temperature (28°C) showed that multiple temperature-sensitive steps were involved in the inhibition of virus replication.

The effects of temperature on the replications of some shrimp viruses are well documented. WSSV, a dsDNA virus, found at 16 – 32 °C in shrimp, crab, and crayfish (Jiravanichpaisal, 2006). This broad temperature range might explain the prevalence of WSSV in the environment. This temperature window (16 - 32 °C) for WSSV susceptibility is about the same with shrimp optimum temperature, the tropical water temperature where shrimp are normally raised. In the natural environment, Otta (1999) reported that WSSV occurs 1-2 months after pond stocking.

Vidal et al. (2001) found that 26-27°C is the optimum temperature for WSSV replication, about the same temperature for the optimum growth of shrimp. Rahman et al. (2006a, 2006b, 2007) also concluded that the optimum temperature for the WSSV replication is 27°C and low replication at 33°C.

Jiravanichpaisal et al. (2004) studied that crayfish could carry WSSV at low temperatures (4 or 12°C) and then developed the infections at higher temperature (22°C). One of the explanations was that WSSV virions might be able to enter host cells and subsequently replicate at higher temperatures, but that the virions can only first bind to the cell surface at lower temperature. Alternatively, the higher temperatures may diminish the host defense mechanism since 22°C is outside crayfish optimum range in the natural environment. Recent findings by Lin et al. (2011) described that both the heat shock protein gene (*hsp70*) and the aldehyde dehydrogenase (ALDH) play important roles in the inhibition of WSSV replication at temperature higher than 32°C.

For another shrimp DNA virus, Montgomery-Brock et al. (2007) found that the replication of infectious hypodermal and hematopoietic necrosis virus (IHHNV), a ssDNA virus, was reduced in warm water (32°C). The optimum temperature for the virus is around 27°C.

Some work has also been published concerning the effects of temperature on Taura syndrome virus (TSV), a positive strand RNA virus, replication in *P. vannamei*. Montgomery-Brock et al. (2004) showed that shrimp held at 27°C had a 30% survival rate, while shrimp held at 30°C had a higher survival rate of 85%. Supplementary research found a reduction in the number of TSV genome copies found post injection for animals held at 32°C, indicating that TSV did not replicate as well in shrimp held at the warmer temperature as it did in shrimp held at the cooler temperature of 25°C.

Considering the effects of temperature on two shrimp DNA models (WSSV and IHHNV) and two an RNA model (TSV), there are some patterns. The DNA viruses have broad temperature range of 16 - 32°C and optimum around 27°C for both viruses. The pathogenicity of dsDNA (WSSV) is higher compared to ssDNA (IHHNV). For TSV, the temperature window is around

25 - 34°C and is optimum at 27°C. There is no published paper on the temperature windows of IMNV. Therefore, further research is needed to confirm if there is a temperature-dependent for replications and infections of IMNV.

The current trend of warming global climate could result in changes in the vital biological properties of many coastal and marine environments. It has been suggested by Harvell et al. (2001) that such climate-mediated physiological pressures may also decrease host resistance and making them more susceptible to disease resulting in an increase in opportunistic diseases.

2. Materials and Methods

2.1.First study

Specific Pathogen Free (SPF) shrimp (average weight of 3 gram) were randomly selected to different groups (26°C, 30°C, and 34°C). Each group was done in duplicate in a 90L aquarium. Each aquarium had 15 animals. Control group was conducted at 30°C and not at other temperatures, based on field observation in Indonesia where IMNV mostly occurred around 28-30°C (unpublished data). Each animal received approximately 10^6 copies/µL of IMNV via intramuscularly injection. The experiment was terminated after 21 days.

2.2. Second study

UAZ-APL received *P. vannamei* (average weight of 2-3 gram) from 30 different family lines from a private breeding company for an IMNV challenge (UAZ case 11-272) at the West Campus Agricultural Center facilities. The unchallenged negative controls were used for the follow-up experiment (this study, UAZ case 11-300). On day 0, all shrimp were caught from the holding tanks and transferred to different control and treatment tanks. A total of 64-65 animals per tank were used in each 1000 L tank.

The IMNV isolate used in the challenge study was from UAZ case 08-026 originally from Brazil, with a known concentration defined by qRT-PCR (8.30 X 10^6 copies/µL RNA). The isolate was stored at -70°C at the University of Arizona Aquaculture Pathology Laboratory (UAZ-APL).

Challenge test

The shrimp in two challenged tanks (26°C and 30°C) were injected with approximately 50 µL of a 1:5 dilution of IMNV 10^6 copies/µL in the 3rd abdominal segment. The other two tanks served as negative controls and were injected with 30 µL of 2% saline. The negative control tanks and challenged tanks were maintained in two different rooms to prevent cross contamination. The rooms were set at (26 ± 1)°C and heaters were put in the (30 ± 1)°C tanks. All shrimp were fed a commercially pelleted diet once a day for the duration of the study.

Histopathology and RT-PCR analysis

Moribund animals were preserved in Davidson's AFA fixative (Bell and Lightner, 1988) to confirm IMNV infection as well as representative from survivors at the termination of the study. On day 5, 10, and 20, samples of shrimp were frozen from both challenged tanks and tested for the presence of IMNV by RT-PCR. Additionally, the shrimp in each tank were checked daily for the white muscle that indicates an IMNV infection. The challenged study terminated after 21 days.

For RT-PCR analysis, total RNA was extracted from cephalothorax and abdomen using the RNAeasy extraction kit (Qiagen) according to the manufacturer's protocols. The concentration of the extracted RNA was determined by measuring the optical density (OD) at 260 nm in a spectrophotometer. RT-PCR for IMNV was carried out according to Poulos and Lightner (2006). The presence of IMNV was confirmed by electrophoresis using 1.5% agarose gel containing 2.5% (v/v) ethidium bromide and visualized under ultraviolet light and digitally photographed by the AlphaImager (Alpha Innotech).

Statistical analysis

Statistical analysis was conducted using SPSS 20.0 program. The survival behavior (pattern) analysis was determined by Kaplan Meier survival analysis followed by overall comparisons and pairwise comparisons using Log Rank (Mantel-Cox), Breslow (Generalised Wilcoxon), and Tarone Ware. In the overall comparisons and pairwise comparisons, *p* value (or Sig. as in the

comparison tables) of less than 0.05 shows a significant difference. By having control group in the overall comparisons, it was expected that there would be a significant difference. Therefore, pairwise comparisons were run to confirm the differences between two groups.

In Kaplan Meier survival analysis, mortality events were defined as ‘completed’ or ‘dead’, while the survivors were termed as ‘censored’. For large samples, Kaplan Meier survival analysis is useful in estimating the overall survival patterns (and indirectly the survivals) for the remaining samples when the observation is terminated. For example, in a challenge study of one thousand shrimp with probiotics treatment, the survival pattern can be estimated by observing the performance of the first one hundred shrimp. The remaining nine hundred shrimp are censored, and the estimated pattern will be provided.

As the experiments presented in this paper was terminated when it was likely no more mortalities would occur, the Kaplan Meier analysis for the survival/mortality behavior was based on actual events, and not an estimated one.

2.3. Third study

The third challenge study was conducted as the findings from the first and second experiments showed that temperatures had different effects on the Indonesian and the Brazilian IMNV isolates. This study used smaller number of animals to compare the Indonesian and the Brazilian isolates. The experiment was run at 26°C and 30°C with respective controls in 90 L aquarium. Each of the six groups consisted of 10 animals. The experiment protocols were the same as the second experiment.

3. Results and Discussions

Final survivals

Table 1 shows that based on the final survivals of shrimp from three different IMNV challenge studies, IMNV infection that caused mortalities in shrimp was optimum at 30°C for both the Indonesian and the Brazilian isolates. However, at low temperature (26°C), the mortalities in Indonesian isolate group was reduced (experiment 1 and 3), and the similar effect was not found for the Brazilian isolate group (experiment 2 and 3). The data from the first experiment showed that reduced mortalities occurred not only at low temperature but also at high temperature (34°C). Based on Kaplan Meier survival analysis, the survival patterns from the first experiment were significantly different between groups (Appendix B).

Table 1. Final survivals of shrimp infected with IMNV after 21 days.

Experiment	Number of animals	Temperatures	Survivals (%)
1	30	30°C (control)	93.3
	30	26°C (IMNV Indonesia)	93.3
	30	30°C (IMNV Indonesia)	0
	30	34°C (IMNV Indonesia)	86.7
2	64	26°C (control)	100
	65	26°C (IMNV Brazil)	13.8
	64	30°C (control)	100
	65	30°C (IMNV Brazil)	18.5
3	10	26°C (control)	100
	10	26°C (IMNV Indonesia)	100
	10	26°C (IMNV Brazil)	20
	10	30°C (control)	90
	10	30°C (IMNV Indonesia)	20
	10	30°C (IMNV Brazil)	10

The animals used for the second experiment using the Brazilian isolate came from unchallenged negative control shrimp population that had been used in a previous study (UAZ case 11-272). The previous study used 30 different families maintained at 28°C with average survivals of

13.92%. The final survivals of UAZ case 11-300 (this study) showed 13.8% for 26°C, and 18.5% for 30°C. Detail mortality data can be found in Appendix A. Even though the final survivals at both temperatures seemed similar, however, based on the Kaplan-Meier survival analysis, the survival patterns in both groups were different at $p < 0.05$ (Appendix C).

At the termination of the study, all survivors were recorded based on the tagging, and it was noticed that the treatment tanks received low survival families. Even though the shrimp were randomly selected to negative and treatment tanks to avoid bias at the beginning of study, only at the end of the experiment, it was found that most of the high survival families from the previous study were stocked into the negative control tanks. This fact may be contributes to the low survivals of shrimp in both treatments.

Histopathology observation confirmed the presence of necrosis muscle (Figure 1) and lymphoid organ spheroid (LOS) in all fixed shrimp, both in the dead shrimp and the survivors (Figure 2). While the dead shrimp showed acute phase, the survivors were more on chronic phase. LOS is a common response in shrimp upon infection to many viral and bacterial pathogens and not specific for IMNV. The lymphoid organ is the most effective organ for filtering and eliminating particulate pathogens (Martin, et al. 1996).

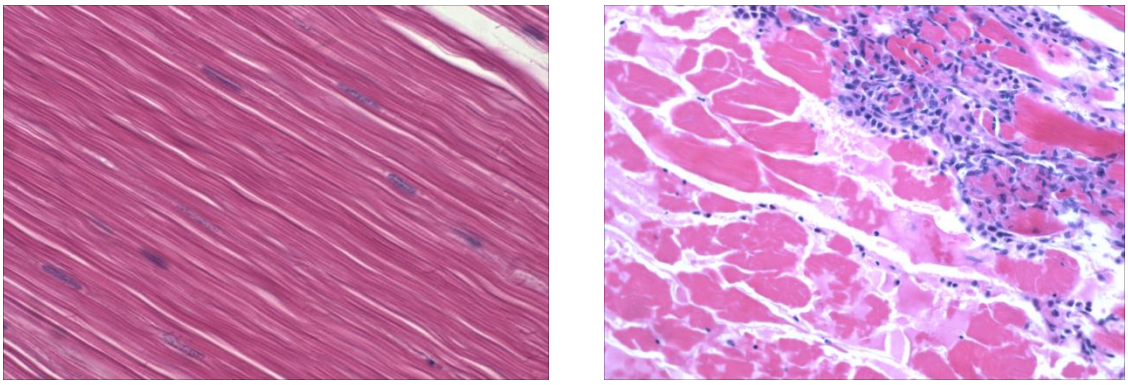


Figure 1. Photomicrograph illustrating normal muscle tissue (left), and acute coagulative necrosis in the muscle fibers due to IMNV (right). The progression of myonecrosis giving affected muscle fiber a hyaline appearance, nuclear pyknosis, edema, and hemocytic infiltration.

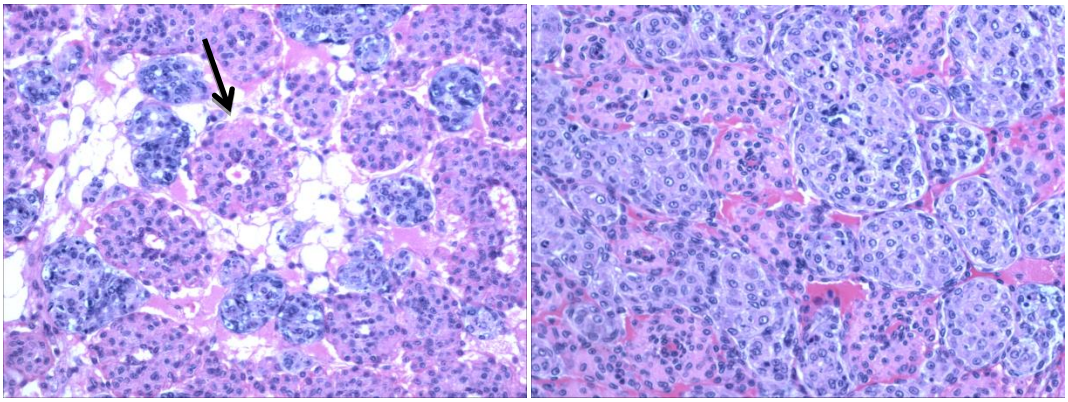


Figure 2. Lymphoid Organ Spheroids of moribund shrimp (left) and survivor (right) at 26°C. A normal arteriole is surrounded by normal LO parenchymal cells present in the field in the left (arrow) for comparison.

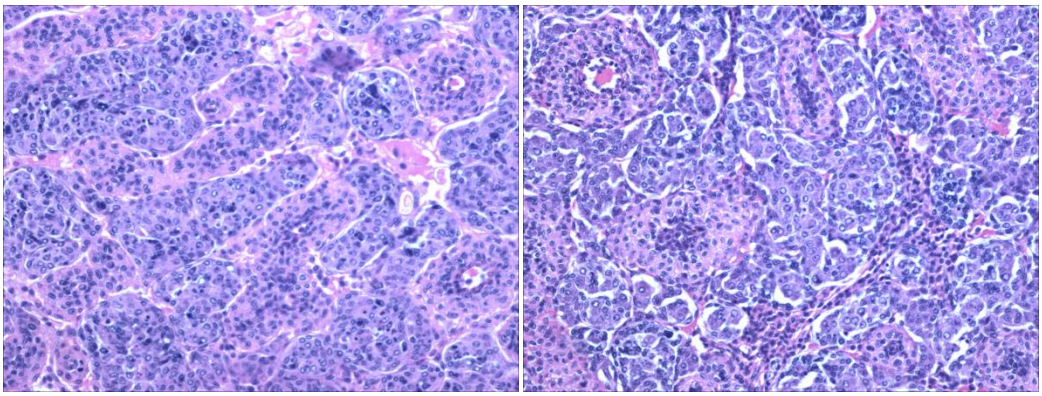


Figure 3. Lymphoid Organ Spheroids of a moribund shrimp (left) and a survivor (right) at 30°C

RT-PCR analysis confirmed the presence of IMNV in dead shrimp and survivors as well. While some survivors and dead animals did not show IMNV with the 1st step RT-PCR, the 2nd step was IMNV positive for all samples with the exception of one sample (Table 2). This finding showed that the second step (nested PCR) is more sensitive than the first step.

Table 2. RT-PCR analysis for moribunds and survivors from a challenge study using IMNV Brazilian (08-026) isolate.

No	Samples	Temperature (°C)	Tissue weight (mg)	RNA (ng/µL)	RNA (260/280)	RT-PCR	
						First step	Second step
1	Control	30	60	210	0.98	negative	negative
2	Survivor	26	41	29	1.96	positive	Positive
3	Survivor	26	64	34	1.23	negative	Positive
4	Survivor	30	60	14	2.11	positive	Positive
5	Survivor	30	58	93	1.25	negative	Positive
6	Survivor	30	58	17	1.50	positive	Positive
7	Survivor	30	70	5	1.24	positive	Positive
8	Survivor	30	67	12	1.35	negative	Positive
9	Survivor	30	65	15	1.35	negative	Negative
10	Survivor	26	34	81	1.06	negative	Positive
11	Moribund (muscle tissue)	30	63	3	1.14	negative	Positive
12	Moribund (muscle tissue)	26	40	7	1.08	negative	Positive
13	Moribund (muscle tissue)	26	75	4	1.43	positive	Positive
14	Moribund (muscle tissue)	30	84	22	1.08	positive	Positive
15	Moribund (muscle tissue)	26	60	111	1.00	positive	Positive
16	Moribund (cephalothorax)	30	80	16	1.83	positive	Positive
17	Moribund (cephalothorax)	26	58	9	1.47	positive	Positive
18	Moribund (cephalothorax)	26	76	9	1.20	positive	Positive
19	Moribund (cephalothorax)	26	55	46	1.01	positive	Positive

The finding from the third experiment confirmed that the IMNV isolates from Indonesia behaved differently at 26 and 30°C, while the Brazilian isolates resulted in similar survival rates at both temperatures. The Indonesian IMNV resulted in no mortality at 26°C. In many places where

shrimp aquaculture is being practiced in Indonesia, temperature of 26°C is considered very rare. It only happens during seasonal change. Most of the time, the temperature in the shrimp farm would be around 28 - 30°C.

Based on three different challenge studies, IMNV infection seems optimum around 30°C, with permissive temperature ranges from at least 26°C to 34°C. Viruses seem to have ability to fit their optimum temperature by following the optimum temperatures for their hosts. For example, the permissive temperature for infectious pancreatic necrotic virus is around 20°C, where salmon is normally being cultured (Roberts & Dobos, 1983). However, as the clinical signs, the histopathology, and the RT-PCR analysis confirmed the IMNV presence in the survivors at different temperatures, the findings suggest that IMNV could bind to the host receptor at low or high temperatures, but caused infections and mortalities in more specific temperature, perhaps around 30°C. Jiravanichpaisal et al. (2004) studied that crayfish could carry WSSV at low temperatures (4 or 12°C) and then developed the infections only at higher temperature (22°C).

Histopathology and RT-PCR analysis also suggest that the survivors might develop some kind of defense mechanism that enables them to minimize the IMNV infections. Therefore, it might be worthy to assess some basic immune parameters for crustacean includes, for example hemograms, percentage of apoptotic hemocytes (Hoechst staining), anion superoxide production (NBT reduction), phenoloxidase (PO) activity (L-DOPA oxidation) (Costa et al., 2009). Furthermore, as RNA viruses bind to the same laminin receptor (Lamr) in shrimp (Busayarat et al., 2011), it would be more interesting to test if IMNV survivors would survive other viral challenge studies (for example TSV or YHV), and *vice versa*. Laminin receptor is the site where the capsid proteins of RNA viruses bind to the host (shrimp). Blocking the binding site of the Lamr may work to block RNA virus infections in shrimp. If Lamr can only bind once to specific RNA virus and the first binding would prevent other RNA viruses, this might explain that routine diagnostic run by the Indonesian research institution for field samples often finds co-infection of WSSV and IHHNV, WSSV and TSV, WSSV and IMNV, but not TSV and IMNV (unpublished data).

What has happened with the natural RNA infections in shrimp in the East Java province, one of central production of shrimp in Indonesia, might give a better description on a viral interference or co-infections study. The first RNA virus infected *P. monodon* in Indonesia was Yellow Head Virus (YHV) in 1993/1994, and localized in Gresik region and then disappeared for unknown explanation in 1995, right after the first case of WSSV found in the region. After the introduction of *P. vannamei* for aquaculture purposes in 2001, two years later (2003/2004) Taura syndrome virus (TSV) outbreak was confirmed close to Gresik region. The spread and economic losses due to TSV is limited mainly because of the availability of post-larvae of specific pathogen resistant (SPR) to TSV. For example, TSV has never been found in Sumatra island, another shrimp central production in Indonesia. Unfortunately, two years later (2005/2006), the first outbreak of IMNV was already confirmed in Situbondo, East Java, not too far from Gresik, where the first cases of YHV and TSV were recorded. By 2011, the disease has spread to 10 out of 17 provinces where *P. vannamei* is being cultured. It is suspected that the virus spread from East Java to South Kalimantan through the movement of infected post larvae. The mode of spread to other provinces, however, is not very clear (Sunarto, 2011).

Why YHV disappeared after WSSV outbreak (both viruses infect *P. monodon* prior to the introduction of *P. vannamei*), or why YHV has never been reported re-emerge in the country after TSV and IMNV introductions (the three viruses are RNA viruses and bind to the same receptor Lamr) are interesting natural facts for further research. It is also interesting, if the limited cases of TSV is mainly due to the availability of SPR for TSV, or if IMNV presence also contributes to the interference because both viruses bind to the same receptor in shrimp.

Conclusion

Based on survivals after challenge studies, IMNV from Brazil caused infections and mortalities in shrimp at 26 and 30°C. IMNV from Indonesia resulted in mortalities in shrimp at 30°C and reduced at 26°C (and also 34°C). Some survivors showed clinical signs of whitish muscle. The survivors from all groups were positive of IMNV by nested PCR following first step RT-PCR. The difference in terms of response to different temperatures in both isolates is unknown and needs further investigations.

References

Allan, E., Froneman, P., & Hodgson, A. (2006). Effects of temperature and salinity on the standard metabolic rate (SMR) of the caridean shrimp *Palaemon peringueyi*. *Journal of Experimental Marine Biology and Ecology*, 337 (1), 103-108.

Busayarat, N., Senapin, S., Tonganunt, M., Phiwsaiya, K., Meemetta, W., Unajak, S., Jitrapakdee, S., Lo, C., & Phongdara, A. 2011. Shrimp laminin receptor binds with capsid proteins of two additional shrimp RNA viruses YHV and IMNV**.** *Fish and Shellfish Immunology, 30* (1), 66-72.

Coelho, M., Silva, A., Vila Nova, C., Neto, J., Lima, A., Feijo, R. (2009). Susceptibility of the wild southern brown shrimp (*Farfantepenaeus subtilis*) to infectious hypodermal and hematopoietic necrosis (IHHN) and infectious myonecrosis (IMN). *Aquaculture, 294* (1-2), 1-4.

Costa, A., Barracco, M., Buglione C., Bezerra F., & Martins P. 2009. Immune assessment of farm-reared *Penaeus vannamei* shrimp naturally infected by IMNV in NE Brazil**.** *Aquaculture, 291* (3-4), 141-146.

Goodman, R., Ghabrial, S., Fichorova, R., & Nibert, M. (2011). Trichomonasvirus: A new genus of protozoan viruses in the family *Totiviridae*. *Archives of Virology, 156* (1), 171-179.

Guan, L., Kawamura, H., & Murakami, H. (2003). Retrieval of sea surface temperature from TRMM VIRS. *Journal of Oceanography, 59* (2), 245-249.

Harvell, D., Kim, K., Quirolo, C., Weir, J., & Smith, G. (2001). Coral bleaching and disease: contributors to 1998 mass mortality in *Briareum asbestinum* (Octocorallia, Gorgonacea). *Hydrobiologia, 460* (1-3), 97-104.

Haugland, O., Mikalsen, A., Nilsen, P., Lindmo, K., Thu, B., Eliassen, T. (2011). Cardiomyopathy syndrome of atlantic salmon (*Salmo salar* L.) is caused by a double-stranded RNA virus of the *Totiviridae* family. *Journal of Virology, 85* (11), 5275-5286.

Jiravanichpaisal, P., Soderhall, K., & Soderhall, I. (2004). Effect of water temperature on the immune response and infectivity pattern of white spot syndrome virus (WSSV) in freshwater crayfish. *Fish & Shellfish Immunology , 17* (3), 265-275.

Jiravanichpaisal, P., Sricharoen, S., Soderhall, I., & Soderhall, K. (2006). White spot syndrome virus (WSSV) interaction with crayfish haemocytes. *Fish and Shellfish Immunology , 20* (5), 718-727.

Lin, Y., Hung, H., Leu, J., Wang, H., Kou, G., & Lo, C. (2011). The role of aldehyde dehydrogenase and hsp70 in suppression of white spot syndrome virus replication at high temperature. *Journal of Virology, 85* (7), 3517-3525.

Liu, H., Jiang, D., Li, G., Fu, Y., Xie, J., Cheng, J. (2010). Widespread horizontal gene transfer from double-stranded RNA viruses to eukaryotic nuclear genomes. *Journal of Virology , 84* (22), 11876-11887.

Martin, G., Hose, J., Minka, G., & Rosenberg, S. 1996. Clearance of bacterial injected into the hemolymph of the ridgeback prawn, *Sicyonia ingentis* (Crustacea: Decapoda): role of hematopoietic tissue. *Journal of Morphology*, 227, 227-233.

Montgomery-Brock, D., Tacon, A., Poulos, B., & Lightner, D. (2007). Reduced replication of infectious hypodermal and hematopoietic necrosis virus (IHHNV) in *Litopenaeus vannamei* held in warm water. *Aqauculture , 265* (1-4), 41-48.

Nibert, M. (2007). '2A-like' and 'shifty heptamer' motifs in penaeid shrimp infectious myonecrosis virus, a monosegmented double-stranded RNA virus. *The Journal of General Virology , 88* (4), 1305-1308.

Otta, S., Shubha, G., Joseph, B., Chakraborty, A., Karunasagar, I., & Karunasagar, I. (1999). Polymerase chain reaction (PCR) detection of white spot syndrome virus (WSSV) in cultured and wild crustaceans in India. *Diseases of Aquatic Organisms , 38*, 67-70.

Poulos, B., & Lightner, D. (2006). Detection of infectious myonecrosis virus (IMNV) of penaeid shrimp by reverse transcriptase polymerase chain reaction (RT-PCR). *Diseases of Aquatic Organisms, 73* (1), 69-72.

Rahman, M., Corteel, M., Dantas-Lima, J., Wille, M., Alday-Sanz, V., Pensaert, M. (2007). Impact of daily fluctuations of optimum (27 °C) and high water temperature (33 °C) on *Penaeus vannamei* juveniles infected with white spot syndrome virus (WSSV). *Aquaculture, 269* (1-4), 107-113.

Rahman, M., Escobedo-Bonilla, C., Corteel, M., Dantas-Lima, J., Wille, M., Alday Sanz, V. (2006a). Effect of high water temperature (33°C) on the clinical and virological outcome of experimental infections with white spot syndrome virus (WSSV) in specific pathogen-free (SPF) *Litopenaeus vannamei*. *Aquaculture , 61* (3), 842-849.

Rahman, M., Escobedo-Bonilla, C., Wille, M., Alday Sanz, V., Audoorn, L., Neyts, J., et al. (2006b). Clinical effect of cidofovir and a diet supplemented with *Spirulina platensis* in white spot syndrome virus (WSSV) infected specific pathogen-free *Litopenaeus vannamei* juveniles. *Aquaculture, 255* (1), 600-605.

Roberts, T., & Dobos, P. 1983. Studies on the mechanism of temperature sensitivity of infectious pancreatic necrosis virus replication. *The Journal of General Virology*, *64* (Pt 2), 330-339.

Sabanadzovic, S., Valverde, R., Brown, J., Martin, R., & Tzanetakis, I. (2009). Southern tomato virus: The link between the families *Totiviridae* and *Partitiviridae*. *Virus Research, 140* (1-2), 130-137.

Senapin, S., Phewsaiya, K., Briggs, M., & Flegel, T. (2007). Outbreaks of infectious myonecrosis virus (IMNV) in Indonesia confirmed by genome sequencing and use of an alternative RT-PCR detection method. *Aquaculture, 266* (1-4), 32-38.

Senapin, S., Phiwsaiya, K., Gangnonngiw, W., & Flegel, T. (2011). False rumours of disease outbreaks caused by infectious myonecrosis virus (IMNV) in the whiteleg shrimp in Asia. *Journal of Negative Results in BioMedicine , 10* (1), 10.

Sunarto, A. (2011). Current status of infectious myonecrosis virus (IMNV) in Pacific white shrimp (*Litopenaeus vannamei*) in Indonesia. *Paper presented in "One Day Workshop on Prevention and Control of IMNV in Indonesia".* Jakarta: Ministry of Marine Affairs and Fisheries. 14 July 2011.

Tang, J., Ochoa, W., Sinkovits, R., Poulos, B., Ghabrial, S., Lightner, D., et al. (2008). Infectious myonecrosis virus has a totivirus-like, 120-subunit capsid, but with fiber complexes at the fivefold axes. *Proceedings of the National Academy of Sciences of the United States of America , 105* (45), 17526-17530.

Tang, K., Pantoja, C., Poulos, B., Redman, R., & Lightner, D. (2005). In situ hybridization demonstrates that *Litopenaeus vannamei*, *L. stylirostris* and *Penaeus monodon* are susceptible to experimental infection with infectious myonecrosis virus (IMNV). *Diseases of Aquatic Organisms, 63*, 261-265.

Wyban, J., Walsh, W., & Godin, D. (1995). Temperature effects on growth, feeding rate and feed conversion of the Pacific white shrimp (*Penaeus vannamei*). *Aquaculture, 138* (1-4), 267-279.

Wu, W., Luo, Y., Lu, R., Lau, N., Lai, E., Li, X., & Ding, S. 2010. Virus discovery by deep sequencing and assembly of virus-derived small silencing RNAs. *Proceedings of the National Academy of Sciences of the United States of America, 107* (4), 1606-1611.

Zhai, Y., Attoui, H., Mohd Jaafar, F., Wang, H., Cao, Y., Fan, S. (2010). Isolation and full-length sequence analysis of Armigeres subalbatus totivirus, the first totivirus isolate from mosquitoes representing a proposed novel genus (Artivirus) of the family *Totiviridae*. *The Journal of General Virology, 91* (11), 2836-2845.

Appendix A: Statistical Analysis

Survivals and number of animals from the 30 family lines in UAZ case 11-272 and 11-300 IMNV studies using Brazilian IMNV isolate (08-026).

Family	UAZ case 11-272 (28°C)		UAZ case 11-300 (30°C)		UAZ case 11-300 (26°C)	
	number of animals	survival rate (%)	number of animals	survival rate (%)	number of animals	survival rates
1	23	8.70	5	0	3	0
2	20	15.00	5	0	4	0
3	23	4.35	4	0	1	0
4	19	15.79	4	25	1	0
5	16	12.50	4	0	1	0
6	21	4.76	5	0	3	0
7	14	0.00	0	0	0	0
8	18	16.67	2	0	2	0
9	16	0.00	6	0	5	0
10	21	0.00	3	0	2	0
11	21	4.76	3	0	1	0
12	19	10.53	9	11.11	5	0
13	17	29.41	0	0	0	0
14	13	46.15	4	0	2	0
15	15	6.67	1	0	1	0
16	15	0.00	5	0	2	0
17	21	0.00	8	0	3	0
18	20	30.00	8	0	3	0
19	19	15.79	7	14.29	4	0
20	20	20.00	8	0	5	0
21	20	0.00	3	0	0	0
22	20	35.00	5	80	3	66.7
23	18	16.67	7	14.29	3	0
24	17	11.76	2	0	2	0
25	22	4.55	2	0	1	0
26	14	35.71	1	100	0	0
27	18	22.22	5	0	1	0
28	20	30.00	4	25	2	50
29	19	10.53	3	100	1	100
30	20	10.00	6	0	4	0

Appendix B

Kaplan-Meier Analysis

Indonesian IMNV isolate study

Case Processing Summary

Group	Total N	N of Events	Censored	
			N	Percent
Control30	30	2	28	93.3%
Treatment30	30	30	0	0.0%
Treatment26	30	2	28	93.3%
Treatment34	30	4	26	86.7%
Overall	120	38	82	68.3%

Survival Table

Group		Time	Status	Cumulative Proportion Surviving at the Time		N of Cumulative Events	N of Remaining Cases
				Estimate	Std. Error		
	1	7.000	dead	.967	.033	1	29
	2	13.000	dead	.933	.046	2	28
	3	21.000	censored	.	.	2	27
	4	21.000	censored	.	.	2	26
	5	21.000	censored	.	.	2	25
	6	21.000	censored	.	.	2	24
	7	21.000	censored	.	.	2	23
Control30	8	21.000	censored	.	.	2	22
	9	21.000	censored	.	.	2	21
	10	21.000	censored	.	.	2	20
	11	21.000	censored	.	.	2	19
	12	21.000	censored	.	.	2	18
	13	21.000	censored	.	.	2	17
	14	21.000	censored	.	.	2	16
	15	21.000	censored	.	.	2	15

	16	21.000	censored	.	.	2	14
	17	21.000	censored	.	.	2	13
	18	21.000	censored	.	.	2	12
	19	21.000	censored	.	.	2	11
	20	21.000	censored	.	.	2	10
	21	21.000	censored	.	.	2	9
	22	21.000	censored	.	.	2	8
	23	21.000	censored	.	.	2	7
	24	21.000	censored	.	.	2	6
	25	21.000	censored	.	.	2	5
	26	21.000	censored	.	.	2	4
	27	21.000	censored	.	.	2	3
	28	21.000	censored	.	.	2	2
	29	21.000	censored	.	.	2	1
	30	21.000	censored	.	.	2	0
	1	3.000	dead	.	.	1	29
	2	3.000	dead	.933	.046	2	28
	3	4.000	dead	.	.	3	27
	4	4.000	dead	.	.	4	26
	5	4.000	dead	.	.	5	25
	6	4.000	dead	.	.	6	24
	7	4.000	dead	.	.	7	23
	8	4.000	dead	.	.	8	22
	9	4.000	dead	.700	.084	9	21
	10	5.000	dead	.	.	10	20
	11	5.000	dead	.	.	11	19
Treatment30	12	5.000	dead	.	.	12	18
	13	5.000	dead	.	.	13	17
	14	5.000	dead	.533	.091	14	16
	15	6.000	dead	.	.	15	15
	16	6.000	dead	.	.	16	14
	17	6.000	dead	.	.	17	13
	18	6.000	dead	.	.	18	12
	19	6.000	dead	.	.	19	11
	20	6.000	dead	.	.	20	10
	21	6.000	dead	.	.	21	9
	22	6.000	dead	.	.	22	8
	23	6.000	dead	.233	.077	23	7

	24	7.000	dead	.	.	24	6
	25	7.000	dead	.	.	25	5
	26	7.000	dead	.	.	26	4
	27	7.000	dead	.100	.055	27	3
	28	8.000	dead	.	.	28	2
	29	8.000	dead	.033	.033	29	1
	30	16.000	dead	.000	.000	30	0
	1	15.000	dead	.967	.033	1	29
	2	20.000	dead	.933	.046	2	28
	3	21.000	censored	.	.	2	27
	4	21.000	censored	.	.	2	26
	5	21.000	censored	.	.	2	25
	6	21.000	censored	.	.	2	24
	7	21.000	censored	.	.	2	23
	8	21.000	censored	.	.	2	22
	9	21.000	censored	.	.	2	21
	10	21.000	censored	.	.	2	20
	11	21.000	censored	.	.	2	19
	12	21.000	censored	.	.	2	18
	13	21.000	censored	.	.	2	17
	14	21.000	censored	.	.	2	16
	15	21.000	censored	.	.	2	15
Treatment26	16	21.000	censored	.	.	2	14
	17	21.000	censored	.	.	2	13
	18	21.000	censored	.	.	2	12
	19	21.000	censored	.	.	2	11
	20	21.000	censored	.	.	2	10
	21	21.000	censored	.	.	2	9
	22	21.000	censored	.	.	2	8
	23	21.000	censored	.	.	2	7
	24	21.000	censored	.	.	2	6
	25	21.000	censored	.	.	2	5
	26	21.000	censored	.	.	2	4
	27	21.000	censored	.	.	2	3
	28	21.000	censored	.	.	2	2
	29	21.000	censored	.	.	2	1
	30	21.000	censored	.	.	2	0
	1	11.000	dead	.967	.033	1	29
Treatment34	2	16.000	dead	.933	.046	2	28
	3	20.000	dead	.900	.055	3	27

4	21.000	dead	.867	.062	4	26
5	21.000	censored	.	.	4	25
6	21.000	censored	.	.	4	24
7	21.000	censored	.	.	4	23
8	21.000	censored	.	.	4	22
9	21.000	censored	.	.	4	21
10	21.000	censored	.	.	4	20
11	21.000	censored	.	.	4	19
12	21.000	censored	.	.	4	18
13	21.000	censored	.	.	4	17
14	21.000	censored	.	.	4	16
15	21.000	censored	.	.	4	15
16	21.000	censored	.	.	4	14
17	21.000	censored	.	.	4	13
18	21.000	censored	.	.	4	12
19	21.000	censored	.	.	4	11
20	21.000	censored	.	.	4	10
21	21.000	censored	.	.	4	9
22	21.000	censored	.	.	4	8
23	21.000	censored	.	.	4	7
24	21.000	censored	.	.	4	6
25	21.000	censored	.	.	4	5
26	21.000	censored	.	.	4	4
27	21.000	censored	.	.	4	3
28	21.000	censored	.	.	4	2
29	21.000	censored	.	.	4	1
30	21.000	censored	.	.	4	0

Means and Medians for Survival Time

Group	Mean[a]				Median			
	Estimate	Std. Error	95% Confidence Interval		Estimate	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound			Lower Bound	Upper Bound
Control30	20.267	.521	19.246	21.287	.	.	.	.
Treatment30	5.767	.430	4.923	6.611	6.000	.330	5.351	6.649
Treatment26	20.767	.198	20.378	21.155	.	.	.	.
Treatment34	20.467	.417	19.649	21.284	.	.	.	.
Overall	16.817	.623	15.595	18.038	.	.	.	.

a. Estimation is limited to the largest survival time if it is censored.

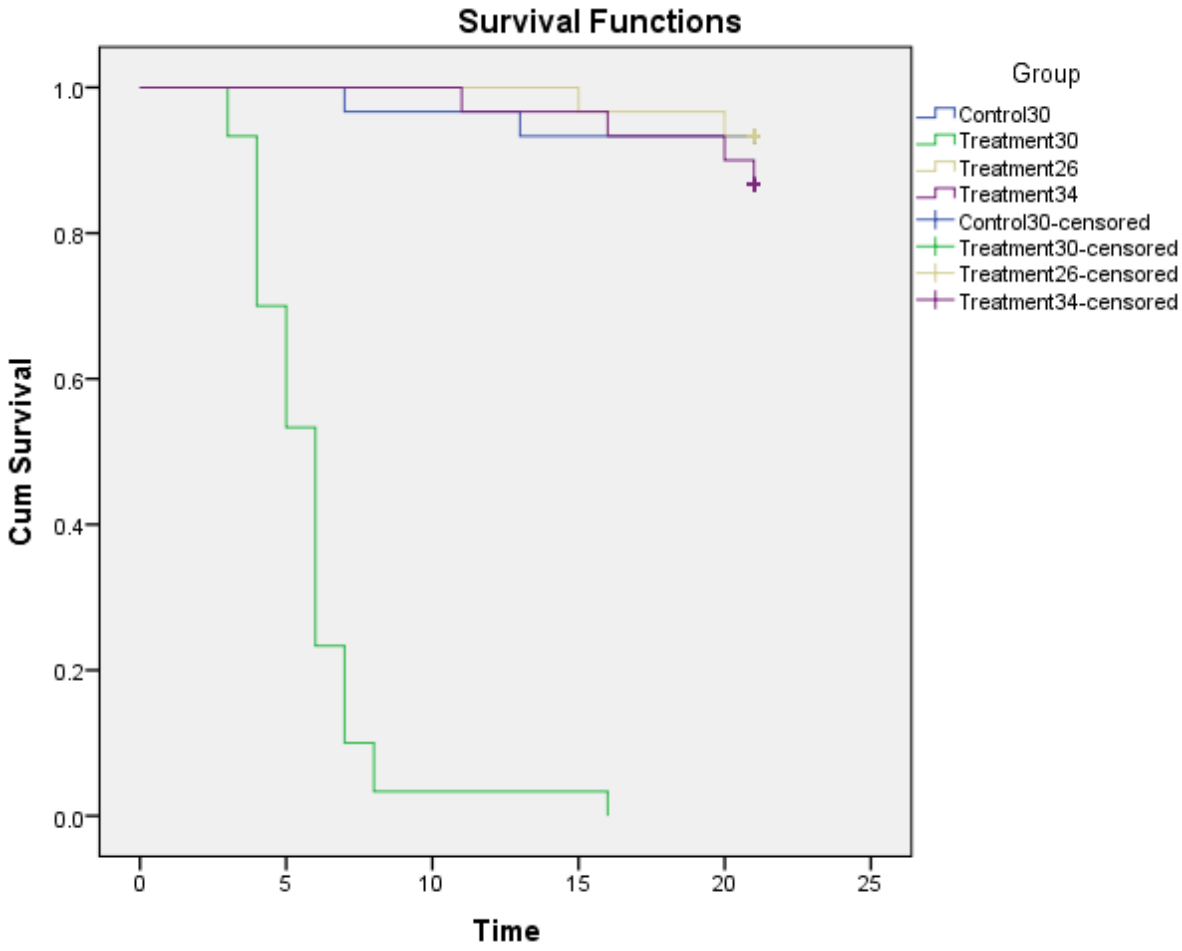
Overall Comparisons

	Chi-Square	df	Sig.
Log Rank (Mantel-Cox)	164.655	3	.000
Breslow (Generalized Wilcoxon)	152.838	3	.000
Tarone-Ware	158.719	3	.000

Test of equality of survival distributions for the different levels of Group.

Pairwise Comparisons

	Group	Control30		Treatment30		Treatment26		Treatment34	
		Chi-Square	Sig.	Chi-Square	Sig.	Chi-Square	Sig.	Chi-Square	Sig.
Log Rank (Mantel-Cox)	Control30			64.201	.000	.001	.972	.656	.418
	Treatment30	64.201	.000			66.986	.000	66.403	.000
	Treatment26	.001	.972	66.986	.000			.719	.397
	Treatment34	.656	.418	66.403	.000	.719	.397		
Breslow (Generalized Wilcoxon)	Control30			53.865	.000	.005	.945	.588	.443
	Treatment30	53.865	.000			55.676	.000	55.449	.000
	Treatment26	.005	.945	55.676	.000			.702	.402
	Treatment34	.588	.443	55.449	.000	.702	.402		
Tarone-Ware	Control30			58.938	.000	.003	.959	.621	.430
	Treatment30	58.938	.000			61.188	.000	60.824	.000
	Treatment26	.003	.959	61.188	.000			.710	.399
	Treatment34	.621	.430	60.824	.000	.710	.399		


Survival Functions
Cum Survival
1.0
0.8
0.6
0.4
0.2
0.0
0
5
10
15
20
25
Time
Group
Control30
Treatment30
Treatment26
Treatment34
Control30-censored
Treatment30-censored
Treatment26-censored
Treatment34-censored

Appendix B

Kaplan-Meier

Brazilian Isolate Study

No statistics are computed because all cases are censored.
No statistics are computed because all cases are censored.

Case Processing Summary

Group	Total N	N of Events	Censored	
			N	Percent
Control30	64	0	64	100.0%
Treatment30	65	53	12	18.5%
Control26	64	0	64	100.0%
Treatment26	65	56	9	13.8%
Overall	258	109	149	57.8%

Survival Table

Group		Time	Status	Cumulative Proportion Surviving at the Time		N of Cumulative Events	N of Remaining Cases
				Estimate	Std. Error		
	1	21.000	censored	.	.	0	63
	2	21.000	censored	.	.	0	62
	3	21.000	censored	.	.	0	61
	4	21.000	censored	.	.	0	60
	5	21.000	censored	.	.	0	59
Control30	6	21.000	censored	.	.	0	58
	7	21.000	censored	.	.	0	57
	8	21.000	censored	.	.	0	56
	9	21.000	censored	.	.	0	55
	10	21.000	censored	.	.	0	54
	11	21.000	censored	.	.	0	53

	12	21.000	censored	.	.	0	52
	13	21.000	censored	.	.	0	51
	14	21.000	censored	.	.	0	50
	15	21.000	censored	.	.	0	49
	16	21.000	censored	.	.	0	48
	17	21.000	censored	.	.	0	47
	18	21.000	censored	.	.	0	46
	19	21.000	censored	.	.	0	45
	20	21.000	censored	.	.	0	44
	21	21.000	censored	.	.	0	43
	22	21.000	censored	.	.	0	42
	23	21.000	censored	.	.	0	41
	24	21.000	censored	.	.	0	40
	25	21.000	censored	.	.	0	39
	26	21.000	censored	.	.	0	38
	27	21.000	censored	.	.	0	37
	28	21.000	censored	.	.	0	36
	29	21.000	censored	.	.	0	35
	30	21.000	censored	.	.	0	34
	30	21.000	censored	.	.	0	33
	32	21.000	censored	.	.	0	32
	33	21.000	censored	.	.	0	30
	34	21.000	censored	.	.	0	30
	35	21.000	censored	.	.	0	29
	36	21.000	censored	.	.	0	28
	37	21.000	censored	.	.	0	27
	38	21.000	censored	.	.	0	26
	39	21.000	censored	.	.	0	25
	40	21.000	censored	.	.	0	24
	41	21.000	censored	.	.	0	23
	42	21.000	censored	.	.	0	22
	43	21.000	censored	.	.	0	21
	44	21.000	censored	.	.	0	20
	45	21.000	censored	.	.	0	19
	46	21.000	censored	.	.	0	18
	47	21.000	censored	.	.	0	17

	48	21.000	censored	.	.	0	16
	49	21.000	censored	.	.	0	15
	50	21.000	censored	.	.	0	14
	51	21.000	censored	.	.	0	13
	52	21.000	censored	.	.	0	12
	53	21.000	censored	.	.	0	11
	54	21.000	censored	.	.	0	10
	55	21.000	censored	.	.	0	9
	56	21.000	censored	.	.	0	8
	57	21.000	censored	.	.	0	7
	58	21.000	censored	.	.	0	6
	59	21.000	censored	.	.	0	5
	60	21.000	censored	.	.	0	4
	61	21.000	censored	.	.	0	3
	62	21.000	censored	.	.	0	2
	63	21.000	censored	.	.	0	1
	1	7.000	dead	.	.	1	64
	2	7.000	dead	.	.	2	63
	3	7.000	dead	.	.	3	62
	4	7.000	dead	.	.	4	61
	5	7.000	dead	.	.	5	60
	6	7.000	dead	.	.	6	59
	7	7.000	dead	.	.	7	58
	8	7.000	dead	.	.	8	57
	9	7.000	dead	.	.	9	56
	10	7.000	dead	.	.	10	55
	11	7.000	dead	.	.	11	54
Treatment30	12	7.000	dead	.	.	12	53
	13	7.000	dead	.	.	13	52
	14	7.000	dead	.	.	14	51
	15	7.000	dead	.769	.052	15	50
	16	8.000	dead	.	.	16	49
	17	8.000	dead	.	.	17	48
	18	8.000	dead	.	.	18	47
	19	8.000	dead	.	.	19	46
	20	8.000	dead	.	.	20	45
	21	8.000	dead	.	.	21	44
	22	8.000	dead	.	.	22	43

23	8.000	dead	.	.	23	42
24	8.000	dead	.	.	24	41
25	8.000	dead	.	.	25	40
26	8.000	dead	.	.	26	39
27	8.000	dead	.	.	27	38
28	8.000	dead	.	.	28	37
29	8.000	dead	.	.	29	36
30	8.000	dead	.	.	30	35
30	8.000	dead	.	.	30	34
32	8.000	dead	.	.	32	33
33	8.000	dead	.	.	33	32
34	8.000	dead	.	.	34	30
35	8.000	dead	.	.	35	30
36	8.000	dead	.	.	36	29
37	8.000	dead	.430	.061	37	28
38	9.000	dead	.	.	38	27
39	9.000	dead	.	.	39	26
40	9.000	dead	.	.	40	25
41	9.000	dead	.	.	41	24
42	9.000	dead	.	.	42	23
43	9.000	dead	.	.	43	22
44	9.000	dead	.	.	44	21
45	9.000	dead	.	.	45	20
46	9.000	dead	.	.	46	19
47	9.000	dead	.	.	47	18
48	9.000	dead	.262	.055	48	17
49	10.000	dead	.	.	49	16
50	10.000	dead	.	.	50	15
51	10.000	dead	.	.	51	14
52	10.000	dead	.200	.050	52	13
53	12.000	dead	.185	.048	53	12
54	21.000	censored	.	.	53	11
55	21.000	censored	.	.	53	10
56	21.000	censored	.	.	53	9
57	21.000	censored	.	.	53	8
58	21.000	censored	.	.	53	7
59	21.000	censored	.	.	53	6
60	21.000	censored	.	.	53	5
61	21.000	censored	.	.	53	4
62	21.000	censored	.	.	53	3

	63	21.000	censored	.	.	53	2
	64	21.000	censored	.	.	53	1
	65	21.000	censored	.	.	53	0
	1	21.000	censored	.	.	0	63
	2	21.000	censored	.	.	0	62
	3	21.000	censored	.	.	0	61
	4	21.000	censored	.	.	0	60
	5	21.000	censored	.	.	0	59
	6	21.000	censored	.	.	0	58
	7	21.000	censored	.	.	0	57
	8	21.000	censored	.	.	0	56
	9	21.000	censored	.	.	0	55
	10	21.000	censored	.	.	0	54
	11	21.000	censored	.	.	0	53
	12	21.000	censored	.	.	0	52
	13	21.000	censored	.	.	0	51
	14	21.000	censored	.	.	0	50
	15	21.000	censored	.	.	0	49
	16	21.000	censored	.	.	0	48
	17	21.000	censored	.	.	0	47
	18	21.000	censored	.	.	0	46
Control26	19	21.000	censored	.	.	0	45
	20	21.000	censored	.	.	0	44
	21	21.000	censored	.	.	0	43
	22	21.000	censored	.	.	0	42
	23	21.000	censored	.	.	0	41
	24	21.000	censored	.	.	0	40
	25	21.000	censored	.	.	0	39
	26	21.000	censored	.	.	0	38
	27	21.000	censored	.	.	0	37
	28	21.000	censored	.	.	0	36
	29	21.000	censored	.	.	0	35
	30	21.000	censored	.	.	0	34
	30	21.000	censored	.	.	0	33
	32	21.000	censored	.	.	0	32
	33	21.000	censored	.	.	0	30
	34	21.000	censored	.	.	0	30
	35	21.000	censored	.	.	0	29
	36	21.000	censored	.	.	0	28
	37	21.000	censored	.	.	0	27

	38	21.000	censored	.	.	0	26
	39	21.000	censored	.	.	0	25
	40	21.000	censored	.	.	0	24
	41	21.000	censored	.	.	0	23
	42	21.000	censored	.	.	0	22
	43	21.000	censored	.	.	0	21
	44	21.000	censored	.	.	0	20
	45	21.000	censored	.	.	0	19
	46	21.000	censored	.	.	0	18
	47	21.000	censored	.	.	0	17
	48	21.000	censored	.	.	0	16
	49	21.000	censored	.	.	0	15
	50	21.000	censored	.	.	0	14
	51	21.000	censored	.	.	0	13
	52	21.000	censored	.	.	0	12
	53	21.000	censored	.	.	0	11
	54	21.000	censored	.	.	0	10
	55	21.000	censored	.	.	0	9
	56	21.000	censored	.	.	0	8
	57	21.000	censored	.	.	0	7
	58	21.000	censored	.	.	0	6
	59	21.000	censored	.	.	0	5
	60	21.000	censored	.	.	0	4
	61	21.000	censored	.	.	0	3
	62	21.000	censored	.	.	0	2
	63	21.000	censored	.	.	0	1
	1	8.000	dead	.	.	1	64
	2	8.000	dead	.	.	2	63
	3	8.000	dead	.	.	3	62
	4	8.000	dead	.	.	4	61
	5	8.000	dead	.	.	5	60
	6	8.000	dead	.	.	6	59
Treatment26	7	8.000	dead	.892	.038	7	58
	8	9.000	dead	.	.	8	57
	9	9.000	dead	.	.	9	56
	10	9.000	dead	.	.	10	55
	11	9.000	dead	.	.	11	54
	12	9.000	dead	.	.	12	53

13	9.000	dead	.	.	13	52
14	9.000	dead	.785	.051	14	51
15	10.000	dead	.	.	15	50
16	10.000	dead	.	.	16	49
17	10.000	dead	.	.	17	48
18	10.000	dead	.723	.056	18	47
19	11.000	dead	.	.	19	46
20	11.000	dead	.	.	20	45
21	11.000	dead	.	.	21	44
22	11.000	dead	.	.	22	43
23	11.000	dead	.	.	23	42
24	11.000	dead	.	.	24	41
25	11.000	dead	.	.	25	40
26	11.000	dead	.600	.061	26	39
27	12.000	dead	.	.	27	38
28	12.000	dead	.	.	28	37
29	12.000	dead	.	.	29	36
30	12.000	dead	.	.	30	35
30	12.000	dead	.	.	30	34
32	12.000	dead	.508	.062	32	33
33	13.000	dead	.	.	33	32
34	13.000	dead	.	.	34	30
35	13.000	dead	.	.	35	30
36	13.000	dead	.	.	36	29
37	13.000	dead	.	.	37	28
38	13.000	dead	.	.	38	27
39	13.000	dead	.	.	39	26
40	13.000	dead	.	.	40	25
41	13.000	dead	.	.	41	24
42	13.000	dead	.	.	42	23
43	13.000	dead	.	.	43	22
44	13.000	dead	.	.	44	21
45	13.000	dead	.	.	45	20
46	13.000	dead	.292	.056	46	19
47	14.000	dead	.	.	47	18
48	14.000	dead	.	.	48	17

49	14.000	dead	.	.	49	16
50	14.000	dead	.230	.052	50	15
51	16.000	dead	.	.	51	14
52	16.000	dead	.200	.050	52	13
53	17.000	dead	.	.	53	12
54	17.000	dead	.169	.047	54	11
55	19.000	dead	.154	.045	55	10
56	20.000	dead	.138	.043	56	9
57	21.000	censored	.	.	56	8
58	21.000	censored	.	.	56	7
59	21.000	censored	.	.	56	6
60	21.000	censored	.	.	56	5
61	21.000	censored	.	.	56	4
62	21.000	censored	.	.	56	3
63	21.000	censored	.	.	56	2
64	21.000	censored	.	.	56	1
65	21.000	censored	.	.	56	0

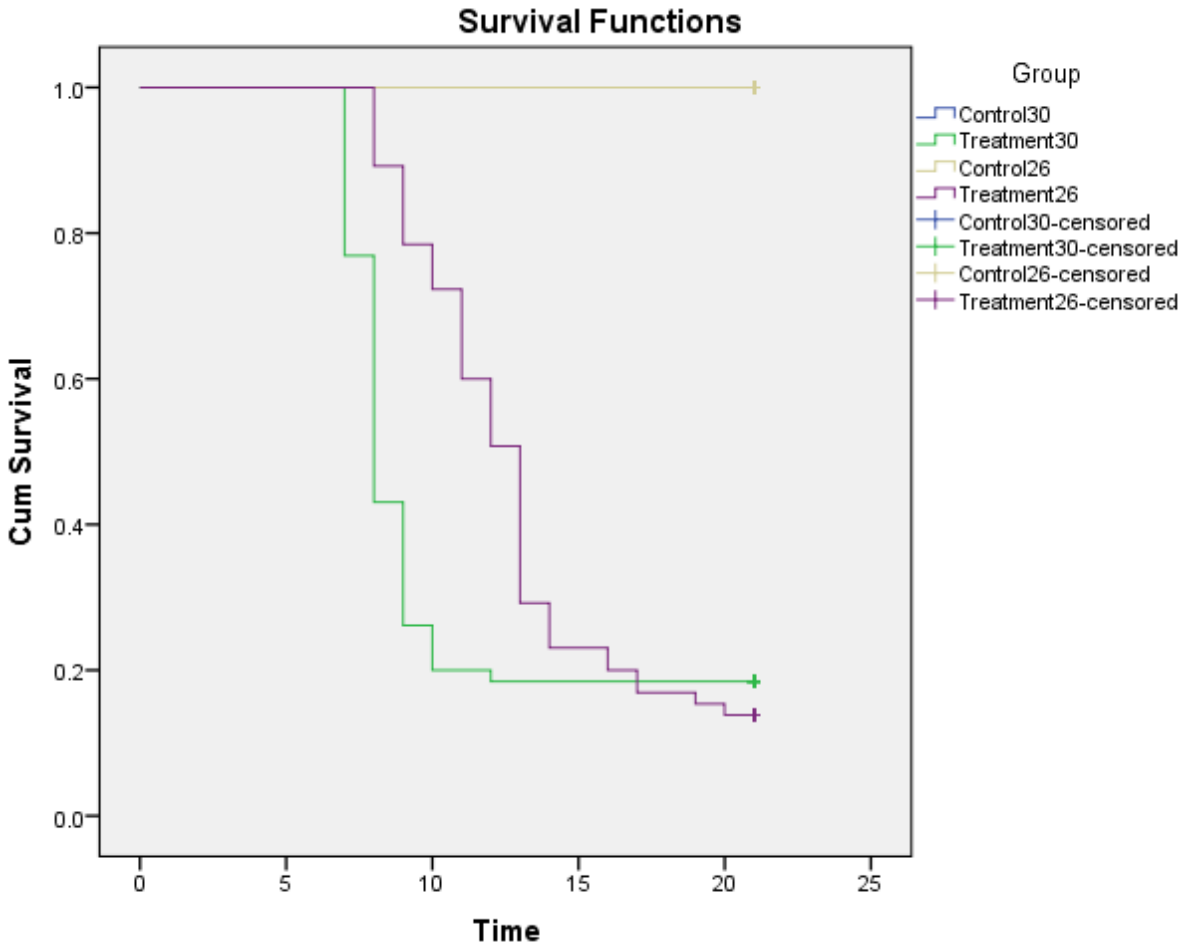
Overall Comparisons

	Chi-Square	df	Sig.
Log Rank (Mantel-Cox)	220.616	3	.000
Breslow (Generalized Wilcoxon)	209.427	3	.000
Tarone-Ware	215.677	3	.000

Test of equality of survival distributions for the different levels of Group.

Pairwise Comparisons

	Group	Control30		Treatment30		Control26		Treatment26	
		Chi-Square	Sig.	Chi-Square	Sig.	Chi-Square	Sig.	Chi-Square	Sig.
Log Rank (Mantel-Cox)	Control30			93.217	.000	.	.	105.090	.000
	Treatment30	93.217	.000			93.217	.000	8.995	.003
	Control26	.	.	93.217	.000			105.090	.000
	Treatment26	105.090	.000	8.995	.003	105.090	.000		
Breslow (Generalized Wilcoxon)	Control30			84.939	.000	.	.	93.712	.000
	Treatment30	84.939	.000			84.939	.000	28.479	.000
	Control26	.	.	84.939	.000			93.712	.000
	Treatment26	93.712	.000	28.479	.000	93.712	.000		
Tarone-Ware	Control30			89.288	.000	.	.	99.583	.000
	Treatment30	89.288	.000			89.288	.000	19.816	.000
	Control26	.	.	89.288	.000			99.583	.000
	Treatment26	99.583	.000	19.816	.000	99.583	.000		


Survival Functions
Cum Survival
1.0
0.8
0.6
0.4
0.2
0.0
0
5
10
15
20
25
Time
Group
Control30
Treatment30
Control26
Treatment26
Control30-censored
Treatment30-censored
Control26-censored
Treatment26-censored